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Characterising changes in pathology and function in clinically relevant models of spinal cord injury and using chondroitinase ABC gene therapy to promote repair

James, Nicholas D.

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**Characterising changes in pathology and function in
clinically relevant models of spinal cord injury and
using chondroitinase ABC gene therapy to promote
repair**

Nicholas D. James

Thesis presented for the degree of

Doctor of Philosophy

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Wolfson Centre for Age Related Diseases

King's College London

University of London

Abstract

Over recent years the bacterial enzyme chondroitinase ABC (ChABC) has emerged as a promising experimental therapeutic for the treatment of spinal cord injury (SCI). In pre-clinical studies ChABC has repeatedly been shown to enhance functional recovery in a number of SCI models in both rodents and larger animals through its enzymatic degradation of inhibitory chondroitin sulphate proteoglycans (CSPGs). However, ChABC treatment has met with limited success in more traumatic, translational models of SCI, such as contusive or compressive injuries. As such injury models mimic the most common form of SCI in humans, it is important to show efficacy of experimental therapeutics in these models. The key aims of this thesis therefore, are to improve upon current methods of ChABC delivery and to assess the efficacy of optimised ChABC in a clinically relevant contusion injury model.

The first series of experiments involved a detailed characterisation of the temporal pattern of functional and anatomical changes that occur following a moderate thoracic contusion injury. Changes in dorsal column sensory axon conduction were associated with early demyelination in the perilesional area and subsequent remyelination mediated primarily by Schwann cells. Further electrophysiological analysis revealed a population of viable dorsal column sensory fibres that remained unable to conduct at chronic post-injury time points, in which conduction could be restored following cooling of the lesion site. This established a reproducible and clinically relevant model, with multiple outcome measures and parameters with which to assess the efficacy of optimised ChABC.

A gene delivery approach was applied to optimise the administration of ChABC. Sustained and widespread CSPG degradation was achieved using a lentiviral vector

containing genetically engineered ChABC (LV-ChABC). This treatment resulted in significant improvements in injury pathology and functional recovery in the moderate thoracic contusion injury model. LV-ChABC treatment resulted in neuroprotection, improved behavioural function, increased spinal conduction through the contusion injury and enhanced plasticity below the level of the injury. Additionally, ChABC gene therapy was associated with modulation of the early post-injury immune response which may have contributed to its effects on neuroprotection, whilst the effect of long-term CSPG degradation were more likely to be responsible for the observed effects on plasticity and spinal conduction.

Since recovery of upper limb function is a top priority for SCI patients, further assessments of LV-ChABC were carried out in a moderate contusion injury performed at the cervical level. This resulted in similar neuroprotective effects and functional recovery. In addition, electrophysiological assessments revealed some improved corticospinal tract function below the level of the injury. Finally, LV-ChABC also resulted in neuroprotection and improved spinal conduction in a more severe model of thoracic contusion injury, illustrating the robust effects of ChABC gene therapy in clinically relevant SCI models of varying severity and at different spinal levels.

Thus, ChABC gene therapy achieves sustained and widespread degradation of growth inhibitory CSPG molecules following a single administration, resulting in significantly improved injury pathology and functional repair of the spinal cord in traumatic, clinically relevant models of spinal contusion injury. If safety issues associated with gene therapy can be addressed and efficacy can be demonstrated in experimental SCI models in larger animals, then ChABC gene therapy represents a promising candidate for clinical translation.

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List of Abbreviations

5-HT	5-hydroxytryptamine
AAV	Adeno-associated virus
AdV	Adenovirus
AIS	ASIA impairment scale
ALS	Amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASIA	American spinal injury association
BBB	Basso, Beattie and Bresnehan (locomotor scale)
BDA	Biotinylated dextran amine
BDNF	Brain-derived neurotrophic factor
BF	Biceps femoris
C-4-S	Chondroitin-4-sulphate
cAMP	Cyclic adenosine monophosphate
CAP-23	Cytoskeleton-associated protein-23
ChABC	Chondroitinase ABC
CMV	Cytomegalovirus
CNS	Central nervous system
CP	Common peroneal
CPG	Central pattern generator
(CS)-GAG	(Chondroitin sulphate)-glycosaminoglycan
CSPG	Chondroitin sulphate proteoglycan
CST	Corticospinal tract
DCN	Dorsal column nucleus
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FLS	Forelimb locomotor scale
GAP-43	Growth-associated protein-43
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GSK-3 β	Glycogen synthase kinase-3 β
HIV	Human immunodeficiency virus
HSPG	Heparan sulphate proteoglycan
HSV	Herpes simplex virus
i.p.	Intraperitoneal
IFN- γ	Interferon- γ
IGF-1	Insulin-like growth factor-1
IGFBP6	Insulin-like growth factor binding protein 6
IH	Infinite Horizon
IL-X	Interleukin (various)
kb	kilobase
kD	Kilodalton
kdyne	kilodyne

KSPG	Keratan sulphate proteoglycan
LAR	Leukocyte common antigen-related phosphatase
LV	Lentiviral vector
MAG	Myelin-associated glycoprotein
MIER	Magnetically-evoked inter-enlargement response
MMP-9	Matrix metalloproteinase-9
mRNA	messenger Ribonucleic acid
MS	Multiple sclerosis
MSC	Multipotent stromal cell
NCAM	Neural cell adhesion molecule
NF200	Neurofilament 200
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NPC	Neural progenitor cell
NSAID	Non-steroidal anti-inflammatory drug
NT-3/4	Neurotrophin-3/4
OEC	Olfactory ensheathing cell
OMgp	Oligodendrocyte-myelin glycoprotein
OPC	Oligodendrocyte precursor cell
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
PirB	Paired immunoglobulin-like receptor B
PNN	Perineuronal net
PNS	Peripheral nervous system
PSA	Polysialic acid
PTP σ	Protein tyrosine phosphatase σ
q-PCR	Quantitative polymerase chain reaction
RAG	Regeneration associated gene
RGC	Retinal ganglion cell
(RM)-ANOVA	(Repeated measures) analysis of variance
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RT	Room temperature
(S)CAP	(Sensory) compound action potential
SCI	Spinal cord injury
SEM	Standard error of the mean
SN	Sural nerve
SSEP	Somatosensory-evoked potential
STAT-3	Signal transducer and activator or transducer-3
TC	Tetracycline
(tcM)MEP	(Transcranial magnetic) motor-evoked potential
TGF- β	Transforming growth factor- β
TNF- α	Tumour necrosis factor α
Trk	Tropomyosin receptor kinase
VEGF	Vascular endothelial growth factor
VSV-G	Vesicular stomatitis virus glycoprotein
XT-1	Xylotransferase-1

Publications arising from this work

Publications:

James ND, Bartus K, Grist J, Bennet DLH, McMahon SB, Bradbury EJ (2011) Conduction failure following spinal cord injury: functional and anatomical changes from acute to chronic stages. *J Neurosci* **31**(50):18543-18555.

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Bartus K, Fricker FR, **James ND**, Zhu N, Garratt AN, Birchmeier C, Bennett DLH, Bradbury EJ (2012) Neuregulin-1 is important for Schwann cell migration and axonal remyelination following spinal cord injury. Soc. Neurosci. Abstr. 270.12.

Didangelos A, Bartus K, **James ND**, Bosch KD, Rogers JH, Schneider BL, Muir EM, Bradbury EJ (2012) Gene delivery of chondroitinase ABC reduces secondary pathology and alters inflammatory events following spinal cord injury. Soc. Neurosci. Abstr. 270.08.

Chapter One

1 CHAPTER 1:

General Introduction

1.1 Spinal cord injury: The clinical issue

Conservative estimates of incidence and prevalence suggest that at least 2.5 million people worldwide are currently living with a spinal cord injury (SCI) (source: International Campaign for Cures of spinal injury Paralysis [ICCP], www.campaignforcure.org) and that there are a further ~180,000 new cases of traumatic SCI per annum (Lee et al., 2013). As well as the dramatic social impact of SCI, the economic cost of injury is also of great significance; with the total direct costs for all causes of SCI in the United States estimated at almost \$8 billion per year (DeVivo, 1997) and at least £500 million in the UK (source: ICCP, www.campaignforcure.org). Whilst motor vehicle accidents and falls are the most common causes of SCI worldwide, aetiology can vary significantly depending on region; in South Africa, for example, violence is the most common cause of SCI (Cripps et al., 2011). In terms of those most at risk, young adult males are by far the most likely to be affected, with males accounting for over 80% of all injuries and nearly half of all injuries occurring between the ages of 16 and 30 (source: National Spinal Cord Injury Statistical Centre [NSCISC], www.nscisc.uab.edu).

In the case of most spinal cord injuries the trauma initially received by the cord can generally be classified as a contusive, compressive and/or a penetrating/lacerative injury (Bunge et al., 1997). Spinal contusion injuries account for over half of all SCIs, whilst compression and laceration injuries account for approximately 20% each (10% are non-traumatic) (Norenberg et al., 2004). The symptoms which manifest post-injury will

determine the exact classification of neurological severity, but SCI will almost invariably result in some form of permanent deficit in sensory, motor and/or autonomic function. The most widely used system for classifying the severity of an injury is that of the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) and this includes scoring using the American Spinal Injury Association (ASIA) Impairment Scale (AIS) (Steeves et al., 2011; Zariffa et al., 2011). There are five categories of neurological function according to the AIS, each corresponding to a letter (A-E). A patient with a classification of AIS-A has no motor or sensory function at the level of the sacral segments 4 or 5, i.e. below the neurological level of injury, this is therefore known as sensorimotor complete SCI. AIS classifications B, C, and D correspond to varying degrees of “incomplete” injury, reflecting some preservation of sensory and/or motor function below the neurological level of injury as well as taking parameters such as muscle strength into account. AIS-E represents normal neurological function. In addition to the obvious sensory, motor and autonomic deficits that are caused by SCI there are often further complications associated, particularly with chronic injury, such as SCI-associated pain and depression (Melzack and Loeser, 1978; Cairns et al., 1996; Siddall et al., 1997; Hoffman et al., 2011).

Despite there being an obvious and significant socioeconomic impact of SCI, there is currently no effective treatment for the condition. Acute clinical management currently revolves around minimising the initial damage caused through immobilisation of the spine as early as possible (i.e. at the scene of the accident) and surgical intervention to perform spinal decompression, spinal realignment, spinal stabilisation and/or clear away bone fragments to prevent them causing further damage. Additionally some patients may receive high-dose steroidal anti-inflammatory treatment (methylprednisolone) in an attempt to attenuate secondary damage, although this is no

longer a recommended standard of care. Initially, the findings of three large-scale multi-centre clinical trials reported that methylprednisolone led to improved functional recovery if administered within 8 hours of injury (Bracken et al., 1984;Bracken et al., 1990;Bracken et al., 1997), the short-time window for efficacy reflecting its expected effects on reducing secondary damage via its anti-inflammatory actions. However, subsequent systematic analysis of the data available from these trials, as well as other available literature, has suggested that the effects of methylprednisolone are negligible at best, as well as raising concerns about the toxicity of the required high doses and the observed pattern of medical complications (Hurlbert, 2000;Coleman et al., 2000;Short et al., 2000;Hurlbert, 2001;Short, 2001). Following an initial recovery phase, patients will participate in a physical rehabilitation program aimed at facilitating and maximising any spontaneous post-injury recovery. Whilst survival rates and functional outcomes are improved by the combination of these strategies, the overall functional benefits are modest at best and there remains a significant and unmet need for therapeutic interventions aimed at promoting functional repair of the injured spinal cord.

1.2 The pathological response to SCI

Pre-clinical modelling of SCI in animals (particularly rodents) has revealed much of the information currently available on the pathological processes which arise following an injury to the spinal cord; this section will therefore focus on data obtained from these pre-clinical studies. Comparisons will be drawn with the observations that have been made in human pathological studies.

At the site of injury the primary mechanical insult to the spinal cord results in immediate death of cells in the near vicinity, including neurons, astrocytes,

oligodendrocytes and endothelial cells, as well as transection of long distance ascending and descending axons and disruption of axonal membranes. As well as the damage and cell death caused by this primary injury, the disruption of endothelial cells leads to localised haemorrhage resulting in compromised delivery of oxygen and key nutrients to the damaged tissue, establishing an ongoing period of haemorrhagic necrosis which is accompanied by the activation of the inflammatory response (Wagner, Jr. et al., 1978; Schwab and Bartholdi, 1996; Norenberg et al., 2004; Hagg and Oudega, 2006). These events trigger the onset of a complex cascade of secondary responses resulting in the progressive spread of damage continuing for months, even years, after the injury itself has occurred in SCI patients. Whilst the timeframes of various aspects of this secondary response to injury often vary between animal models and humans (e.g. axonal degeneration is much more protracted in humans whilst the processes of haemorrhage and angiogenesis occur over a similar timescale in both rodents and humans), many of the key processes involved in secondary damage occur in both rodents and humans (Kakulas, 1999; Norenberg et al., 2004; Buss et al., 2004; Kakulas, 2004; Hagg and Oudega, 2006), making rodents an appropriate system for modelling SCI. A number of the key processes of secondary injury and their effects on endogenous repair are summarised in Figure 1.1.

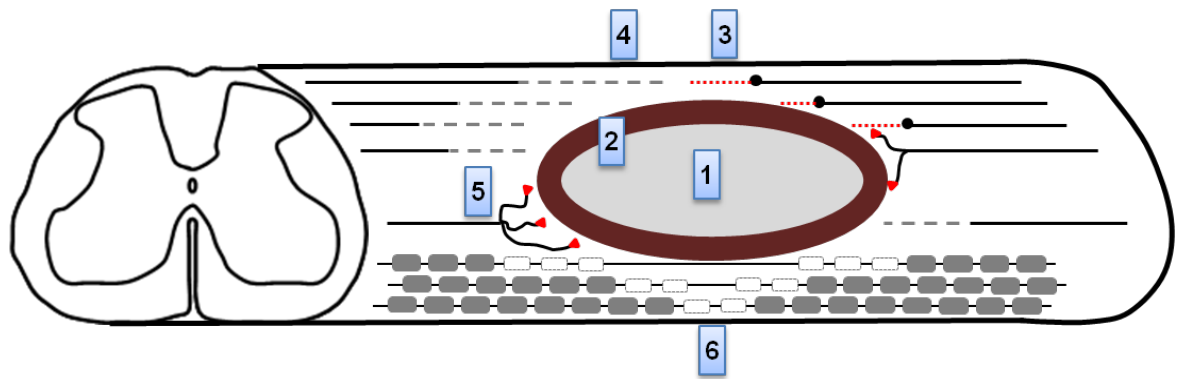


Figure 1.1. Schematic representation of the pathophysiological processes associated with spinal cord injury. 1) Formation of a fluid filled cavity following mass tissue damage and removal due to traumatic lysis, haemorrhage, ischaemia, excitotoxicity and pronounced inflammatory responses. 2) Formation of a glial scar composed primarily of reactive astrocytes and fibroblasts to seal off the damaged tissue, limit spread of damage and restore blood/spinal cord barrier. The glial scar also becomes a physical and chemical barrier to effective repair. 3) Axon segments proximal to the neuronal cell body retract from the injury site, forming dystrophic endbulbs due to microtubule destabilisation. 4) Wallerian degeneration of distal axon segments and slow clearance of cellular debris results in the presence of a number of factors inhibitory to repair. 5) Some sprouting of injured axons occurs, but regeneration fails due to the physical and chemical barrier of the glial scar, numerous extrinsic inhibitory factors and limited intrinsic capacity for growth. 6) Focal demyelination of spared axons in perilesional areas due to oligodendrocyte cell death and shearing of myelin sheaths, resulting in compromised axonal conduction.

1.2.1 Vascular and inflammatory response

Injury related disruption of the spinal cord vasculature results in a number of post-injury complications including ischaemia, oedema and a breakdown in the blood-spinal cord barrier. The lack of vital components needed for metabolism, such as oxygen and glucose, associated with ischaemic conditions has been shown to induce apoptosis

(rather than the necrosis due to primary injury and haemorrhage) (Hayashi et al., 1998; Tanaka et al., 2005; Hagg and Oudega, 2006). The increased production of reactive oxygen species in ischaemic conditions also contributes to substantial tissue loss via cytotoxicity (Xu et al., 2005). Mitochondrial dysfunction has been implicated as a further mechanism underlying apoptosis, due to mitochondrial release of pro-apoptotic proteins following reoxygenation (Tanaka et al., 2005). The increased permeability of damaged vessels following SCI results in oedema (Tator and Fehlings, 1991; Tator, 1991). This swelling of injured spinal tissue can lead to compression of the cord and further damage due to the relative inflexibility of the pial membrane (Norenberg et al., 2004). In addition to mechanical stress, the upregulation and activity of extracellular proteases (particularly matrix metalloproteinase-9 [MMP-9]) is thought to contribute to increased vascular permeability, resulting in a disruption of the blood-spinal cord barrier (Noble et al., 2002). This breakdown of the barrier allows the infiltration of inflammatory cells, augmenting the response of resident immune cells and contributing to the complex neuroinflammatory reaction that is triggered by SCI.

Microglia are the resident immune cells of the central nervous system (CNS) and as such are the first to respond to injury, taking on an “activated” phenotype and releasing pro-inflammatory chemokines and cytokines. The disruption of the blood-spinal cord barrier means that these pro-inflammatory molecules are able to recruit large numbers of infiltrating neutrophils at a very early stage (maximal 12-24 hours post-injury) (Beck et al., 2010). The release of cytokines (such as tumour necrosis factor- α [TNF α] and interleukin-1 β [IL-1 β]) as well as MMP-9 further enhances vascular permeability, exacerbating the influx of circulating inflammatory cells, and can be toxic to both neurons and glia (Donnelly and Popovich, 2008; David and Kroner, 2011). Soon after the infiltration of neutrophils reaches its peak, circulating monocytes are also recruited

to the injury site and these, along with resident microglia, differentiate into pro-inflammatory M1 macrophages which have been shown to exert neurotoxic effects (Kigerl et al., 2009). Lymphocytes are also gradually recruited to the injury site, peaking at around 7-10 days post-injury, reacting against neuroantigens and in particular myelin proteins. This autoimmune response has been shown to amplify the CNS macrophage response and lead to neuronal loss and demyelination (Jones et al., 2002; Jones et al., 2004; Ankeny and Popovich, 2007). Whilst this acute stage of neuroinflammation is vital for restoring tissue homeostasis, it appears to primarily exacerbate secondary damage. A second phase of cellular inflammation however, begins at around 14 days post-injury and during this second phase of the response immune cells are thought to carry out a more reparative function (Kigerl et al., 2009; Beck et al., 2010; David and Kroner, 2011). Macrophages in particular appear to have a dual role in spinal cord injury and repair, displaying markedly different phenotypes depending upon how they have been activated (Kigerl et al., 2009; David and Kroner, 2011). Interferon- γ (IFN γ) polarises macrophages towards a pro-inflammatory M1 phenotype, stimulating them to produce inflammatory cytokines, such as TNF α and IL-1 β , and cytotoxic mediators, such as ROS, whilst IL-4 polarises macrophages towards an alternatively-activated M2 phenotype, inhibiting the production of pro-inflammatory cytokines whilst stimulating the release of anti-inflammatory cytokines, such as IL-10 (Standiford et al., 1990; Tanaka et al., 1993; Paludan, 1998; David and Kroner, 2011). Due to this potential for macrophages to display different phenotypes they are capable of exerting either destructive and degenerative effects or protective and reparative effects. Indeed, many components of the neuroinflammatory response release neuroprotective factors as well as growth factors (Banati and Graeber, 1994; Elkabes et al., 1996; Kerschensteiner et al.,

1999;Nimmerjahn et al., 2005;Kim and de Vellis, 2005), indicating that although inflammation is a major cause of secondary damage, components of it are also vital for the stabilisation of the injury site and can even stimulate regeneration of injured axons.

1.2.2 Excitotoxicity

The post-injury accumulation of excitatory amino acids, particularly glutamate, is yet another cause of secondary damage. Glutamate excitotoxicity can lead to cell death of both glial and neuronal cells due to over-activation of AMPA, kainate and NMDA receptors (Park et al., 2004;Matute, 2011), resulting in lysis due to depolarisation induced high intracellular sodium (Matyja et al., 2005) or apoptosis (Verkhratsky et al., 1998;Matute et al., 2006). Glutamate reaches excitotoxic levels almost immediately after injury (Liu et al., 1991;Farooque et al., 1996;Liu et al., 1999), primarily due to release from lysed and necrotic cells at this early time point (Park et al., 2004). Glutamate continues to be present at excitotoxic levels beyond this initial peak however, and this is thought to be caused by compromised glutamate re-uptake (Mills et al., 2001a;Mills et al., 2001b;Pitt et al., 2003), reversed operation of glutamate transporters resulting in release of glutamate from intracellular stores (McAdoo et al., 2000;Li and Stys, 2001), and high extracellular calcium levels resulting in further glutamate release (LoPachin et al., 1999;Li and Stys, 2001). Excitotoxicity-induced apoptosis not only leads to the loss of neuronal cell bodies in the grey matter, but also the loss of support cells in the white matter, particularly oligodendrocytes, which can lead to disruption in axonal function and even axonal degeneration.

1.2.3 Degeneration and demyelination

The damage inflicted on spinal axons following SCI is a key determinant of the functional consequences that will be suffered. Many axons will be completely severed at the site of injury following mechanical trauma, the distal segments of these axons will

undergo Wallerian degeneration and whilst the proximal segments will generally survive, many of them will withdraw from the injury site and remain there, typically unable to regenerate through or around the lesion (Becerra et al., 1995; Schwab and Bartholdi, 1996). Many axons will form dystrophic end bulbs once they retract from the injury site (Hill et al., 2001; Horn et al., 2008) and further damage can be caused by these structures as upon rupture they will release enzymes, leading to damage of the surrounding tissue (Hagg and Oudega, 2006). In addition to axonal retraction and limited growth capacity, the axotomised cell bodies of many CNS neuronal populations will undergo atrophy (Tetzlaff et al., 1991; Barron, 2004), reflecting a relative lack of trophic support in the injured spinal cord (Widenfalk et al., 2001; Plunet et al., 2002), a failure to upregulate regeneration associated genes (Plunet et al., 2002; Mason et al., 2003) and a decline in the synthesis of vital cytoskeletal proteins including actin and tubulin (Tetzlaff et al., 1991).

In addition to the axotomy of many spinal axons, it is likely that a significant proportion of spared axons within peri-lesional areas will be compromised or damaged despite remaining intact. Depending on the degree of damage, these axons are unlikely to function properly, adding to the functional deficits caused by axotomy. The loss of vascular and glial support for many surviving or injured axons can lead to their gradual degeneration and further tissue loss (Hagg and Oudega, 2006). Additionally, damage to the axonal membrane can lead to ionic imbalance, ultimately resulting in the activation of caspases and exacerbation of existing damage (Bao and Liu, 2003; Wingrave et al., 2003; Iwata et al., 2004). The greatest loss of function in surviving axons is potentially not caused by damage to the axons themselves, but by damage of supporting glial cells, oligodendrocytes in particular. Not only are all types of glial cell capable of providing some form of trophic support for axons (Giehl et al., 1998; Dougherty et al.,

2000;Wilkins et al., 2003;Markiewicz and Lukomska, 2006), but the loss of myelinating oligodendrocytes can lead to conduction block in axons that become focally demyelinated through the lesion site (McDonald and Sears, 1970a;Blight and Young, 1989;Sun et al., 2012). The occurrence of demyelination at the injury site is well established in humans as well as animal models of SCI (Blight, 1983;Crowe et al., 1997;Norenberg et al., 2004;Guest et al., 2005;Totoiu and Keirstead, 2005). In addition to compromising function, demyelination is associated with further axonal degeneration (Irvine and Blakemore, 2008). Demyelination is particularly evident in the acute stages of SCI, and whilst controversy remains about the importance and even the existence of chronic demyelination in rodent models of SCI (discussed in chapter two) (Totoiu and Keirstead, 2005;Lasiene et al., 2008;James et al., 2011;Powers et al., 2012), there can be no question that the necrosis and apoptosis of oligodendrocytes contributes significantly to secondary damage of the spinal parenchyma and ultimately loss of function.

1.2.4 Glial scar and cavity formation

One of the many reactive changes that take place post-SCI is the formation of scar tissue around the injury site to seal in the wound. This is often referred to as the glial scar due the prominent role of reactive astrocytes in its formation, although recent findings have suggested that cells of a pericyte origin also play a significant role (Goritz et al., 2011). Experimental reduction of either cell type leads to ineffective sealing of the wound, indicating that both play a vital role in effective scar formation (Bush et al., 1999;Faulkner et al., 2004;Okada et al., 2006;Herrmann et al., 2008;Goritz et al., 2011). In response to injury, resident spinal cord astrocytes located close to the injury site undergo hypertrophy, proliferation and upregulation of the intermediate filaments glial fibrillary acidic protein (GFAP), vimentin and nestin in a process termed reactive astrogliosis (Pekny et al., 1999;Pekny and Nilsson, 2005;Karimi-Abdolrezaee and

Billakanti, 2012). This is triggered via activation of signalling pathways such as signal transducer and activator of transcription-3 (STAT-3) (Herrmann et al., 2008) and transforming growth factor- β (TGF- β) (Logan et al., 1999b;Schachtrup et al., 2010). Reactive astrocytes at the injury site also release numerous factors such as TGF- β , IL-1 β and IL-6 which lead to the activation and migration of further astrocytes, resulting in densely packed reactive astrocytes with overlapping processes forming a glial scar around the lesion epicentre (Kang and Hebert, 2011;Karimi-Abdolrezaee and Billakanti, 2012).

The presence of the glial scar has both positive and negative effects. The dense glial scar formed around the damaged and dying tissue at the injury site helps to contain the cytotoxic inflammatory response within this injured area, re-establishes the blood-spinal cord barrier, protects against glutamate excitotoxicity via glutamate uptake, and releases anti-oxidants which help defend against oxidative stress (Rothstein et al., 1996;Bush et al., 1999;Fitch et al., 1999;Sofroniew, 2005;Bradbury and Carter, 2011;Karimi-Abdolrezaee and Billakanti, 2012). The damaged area contained within the scar continues to undergo necrotic and apoptotic processes, resulting in large quantities of tissue debris which is gradually phagocytosed, leaving behind a fluid filled cavity surrounded by a dense glial scar (Schwab and Bartholdi, 1996;Fitch et al., 1999;Fitch and Silver, 2008). Genetic ablation of reactive astrocytes has been shown to lead to prolonged failure of the blood-spinal cord barrier, prolonged and widespread inflammation, increased tissue damage and ultimately, decreased functional recovery (Bush et al., 1999;Faulkner et al., 2004). The early formation of a physical barrier of reactive astrocytes around the lesion site therefore appears vital in limiting secondary damage post-SCI. Once local homeostasis is re-established however, the detrimental role of reactive astrogliosis becomes more apparent. As well as providing a physical

barrier to axonal regeneration, reactive astrocytes release a number of factors which inhibit axonal growth and successful cell replacement, resulting in a significantly negative impact on endogenous repair mechanisms post-SCI (Reier et al., 1983; Silver and Miller, 2004; Fitch and Silver, 2008; Wang et al., 2011b). A vast number of growth-inhibitory factors are up-regulated in the extracellular matrix (ECM) following SCI and reactive astrocytes contribute significantly to this, perhaps most significantly of all by releasing chondroitin sulphate proteoglycans (CSPGs). CSPGs are known to be one of the major inhibitors of CNS outgrowth and successful spinal cord repair (Snow et al., 1990; McKeon et al., 1991; McKeon et al., 1995; Bradbury et al., 2002; Barritt et al., 2006; Massey et al., 2008). Along with other factors released by astrocytes, such as insulin-like growth factor binding protein 6 (IGFBP6), CSPGs can also inhibit the differentiation of neural progenitor cells (NPCs) and oligodendrocyte precursor cells (OPCs) into their mature form, thereby preventing the replacement of cells lost due to injury (Barkho et al., 2006; Siebert and Osterhout, 2011; Siebert et al., 2011). So whilst the early response of astrocytes to contain the lesion site prevents further spread of damage, their continued presence around the injury inhibits the already limited endogenous repair mechanisms of the adult spinal cord. The reactive processes that take place following SCI and result in the formation of a glial scar are summarised in figure 1.2.

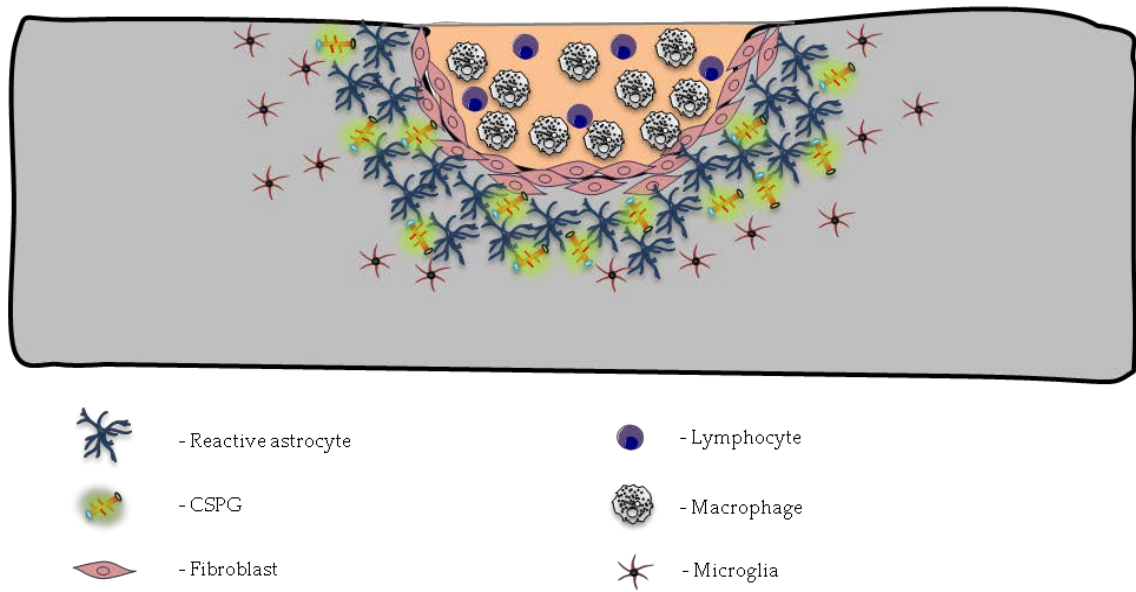


Figure 1.2. Reactive processes at the injury site. Reactive astrocytes and fibroblasts seal off the injury site, forming glial and fibrotic scar tissue. Fibroblasts can be recruited from meningeal (if the meninges are disrupted) (Abnet et al., 1991; Shearer et al., 2003) or perivascular sources (if meninges remain intact, e.g. contusion injury) (Soderblom et al., 2013). Reactive astrocytes secrete inhibitory CSPGs resulting in a dramatic increase in CSPG levels throughout the injury site. Circulating macrophages and lymphocytes, as well as resident microglia, are recruited to the injury site.

1.3 Spontaneous processes of recovery and repair

Despite the combination of significant tissue damage and loss, due to the primary mechanical trauma and the spread of secondary injury processes, and the strongly inhibitory environment of the injured CNS, some degree of spontaneous neurological recovery is associated with SCI in patients as well as in experimental animal models of SCI (Weidner et al., 2001; Fawcett et al., 2007; Courtine et al., 2008; Rosenzweig et al., 2010; Steeves et al., 2011; Zariffa et al., 2011). Recovery of ionic homeostasis as well as

remyelination of demyelinated spared fibres is thought to play a role in mediating this spontaneous recovery (Raineteau and Schwab, 2001;Hagg and Oudega, 2006), but spontaneous compensatory “plasticity” (or reorganisation) of intact or spared circuitry is likely to be the main contributor (Topka et al., 1991;Weidner et al., 2001;Wall et al., 2002;Bareyre et al., 2004;Courtine et al., 2008;Rosenzweig et al., 2010). In humans this has been highlighted by reorganisation of the sensorimotor cortex in SCI patients (Wall et al., 2002), indicated by extensive changes in cortical activation areas during hand movements (Bruehlmeier et al., 1998) and increases in the size of cortical motor representation areas, as well as enhanced excitability of intact motor systems (Levy, Jr. et al., 1990;Topka et al., 1991). Similar changes in cortical organisation have been observed in non-human primate and rodent models of SCI (Fouad et al., 2001;Wall et al., 2002;Bareyre et al., 2004;Jain et al., 2008;Kaas et al., 2008). In addition to observations of functional changes in cortical organisation, SCI studies in animal models have been able to provide anatomical data to further implicate plasticity in the process of spontaneous recovery. For example, in non-human primates that had received a hemisection injury, substantial sprouting of corticospinal tract (CST) axons was observed caudal to the cervical injury site and this was significantly associated with improved function (Rosenzweig et al., 2010). The sprouting primarily occurred in spared CST axons which descended into the spinal cord ipsilaterally before decussating caudal to the injury site. The injury-induced sprouting of these spared axons resulted in the reconstitution of much of the lost CST. Similarly, in rodents lesioning of the main component of the CST can lead to significant compensatory sprouting in the spared minor components of the CST (ventral and dorsolateral), which is associated with spontaneous functional recovery (Weidner et al., 2001;Bareyre et al., 2004) and can be abolished following transection of the spared ventral CST (Weidner et al., 2001).

Anatomical plasticity has been shown to lead to the reorganisation of spinal circuitry, resulting in the formation of compensatory detour pathways (Weidner et al., 2001; Courtine et al., 2008; Vinit et al., 2008; Lang et al., 2012). Despite the spontaneous plasticity that can occur in the injured CNS, neurological recovery is still very much limited in SCI patients and there, therefore, remains a significant need for the development of therapeutic interventions. Knowledge of the spontaneous repair processes that take place in the injured spinal cord is of great benefit in the attempt to develop such therapeutics, as it allows researchers to target potential interventions aimed at enhancing endogenous repair mechanisms rather than trying to instigate *de novo* repair or regeneration. Better understanding of the molecules and mechanisms that limit endogenous repair after CNS injury can lead to the identification of therapeutic targets and will be discussed in the following sections.

1.4 Limited regenerative capacity of the adult spinal cord

The limited capacity for functional recovery and repair following a SCI is primarily due to the inability of the CNS to mount a significant regenerative response to restore connectivity of lesioned spinal pathways. This marks a stark contrast to the robust regenerative response that is launched in response to injury in the peripheral nervous system (PNS), which can often result in significant functional recovery (Fenrich and Gordon, 2004; Huebner and Strittmatter, 2009; Giger et al., 2010). A number of factors contribute to the limited regenerative capacity of the CNS, most of which fall into two broad categories: factors limiting the intrinsic growth capacity of CNS neurons and extrinsic inhibitory factors present in the injured spinal cord.

1.4.1 Intrinsic growth limiting factors

Whilst many studies have now shown that some CNS neurons retain the capacity to regenerate when provided with a more growth permissive environment, such as that of a peripheral nerve graft or cell transplant (Richardson et al., 1980;David and Aguayo, 1981;Cheng et al., 1996;Li et al., 1997;Houle et al., 2006), it is also apparent that many regenerate only feebly whilst others show no regenerative response at all (Grill et al., 1997a;Grill et al., 1997b;Hollis et al., 2009b). The pattern of gene regulation and protein synthesis in axotomised neurons is a key mechanism responsible for this. This becomes particularly apparent when comparisons are made with the response to injury in neurons axotomised in the PNS, where a number of regeneration-associated genes (RAGs) are significantly up-regulated very early after injury (Fenrich and Gordon, 2004;Huebner and Strittmatter, 2009). These up-regulated RAGs include the genes which transcribe the cytoskeletal proteins, tubulin and actin, and the growth-associated proteins, GAP-43 and cytoskeleton-associated protein-23 (CAP-23), both of which have been shown to be important mediators of growth cone elongation (Tetzlaff et al., 1991;Strittmatter et al., 1994;Igarashi et al., 1995;Bomze et al., 2001;Bulsara et al., 2002). Most RAGs are expressed highly in the developing nervous system and then down-regulated in the adult and, whilst peripheral nerve injury triggers robust re-expression of these genes, SCI triggers re-expression in only a fraction of the injured neurons (Skene, 1989;Tetzlaff et al., 1991;Fernandes et al., 1999;Bulsara et al., 2002). The comparatively low level of RAG re-expression in the injured CNS is further compounded by inefficient local protein synthesis and degradation. Axons in the PNS have been shown to contain all of the necessary equipment for local protein synthesis and degradation in abundance, such as mRNAs, ribosomal proteins and Golgi-like structures (Gaete et al., 1998;Court FA et al., 2008;Gumy et al., 2010;Bradke et al., 2012), whereas the levels of the same

molecules and proteins in axons of some CNS neuronal populations (e.g. retinal ganglion cells) have been shown to be much lower (Verma et al., 2005). Further study of the levels of proteins and molecules necessary for local translation is yet to be carried out in many other CNS populations. The presence of the components necessary for local protein synthesis and degradation allows the rapid formation of growth cones as well as aiding in their elongation and guidance; low levels of these components in CNS axons would certainly contribute to their limited intrinsic growth capacity.

Another difference between the PNS and CNS is observed in the response of microtubules to axotomy. Following injury in the CNS microtubules depolymerise at the axon stump, meaning they can no longer propel axon growth, which leads to the formation of stereotypical retraction bulbs (Erturk et al., 2007;Bradke et al., 2012). This does not happen in the PNS however, where microtubules are organised and dynamic at the axon stump, aiding in rapid growth cone formation and axon extension (Erturk et al., 2007). Furthermore, stabilisation of microtubules in injured CNS axons leads to growth cone formation and stimulates axon extension (Erturk et al., 2007;Hellal et al., 2011;Sengottuvel et al., 2011), providing further evidence that injury induced depolymerisation and disorganisation of microtubules limits the growth capacity of CNS neurons.

A final intrinsic mechanism that may limit growth in the injured adult CNS is the expression and activation of various receptors. The expression of high affinity neurotrophin receptors, tropomyosin receptor kinases (Trk), provides a significant example of this. Following either spinal hemisection (King et al., 2000) or contusion injury (Liebl et al., 2001), expression of the full forms of TrkA, TrkB and TrkC are lost at the injury site. Axotomised CST neurons maintain TrkB expression at the level of the cell body (Giehl and Tetzlaff, 1996) but appear to lack axonal expression (Lu et al.,

2001). Consistent with this, there is a loss of TrkB expression on injured rubrospinal axons over time (Kwon et al., 2004). However, there is upregulation of a truncated form of TrkB at the borders of the lesion and it is suggested that this sequesters neurotrophins, thereby further restricting their availability within the injured spinal cord (King et al., 2000; Liebl et al., 2001; Widenfalk et al., 2001). The binding of neurotrophins to Trk receptors enhances cell survival, regulates the advance of growth cones and promotes neurite outgrowth. Thus, the loss of this interaction due to reduced receptor expression will clearly have a negative impact on axonal survival and growth. Integrins are another receptor type which appear to be negatively impacted by axonal injury in the CNS. The integrin family of receptors bind to ECM molecules, such as laminin, fibronectin, collagen and tenascin, and their presence on axons therefore allows axonal elongation over ECM components (Eva et al., 2012). Injury in the PNS induces increased expression of numerous integrin subunits (Hammarberg et al., 2000; Wallquist et al., 2004), and experimental depletion of specific integrin subunits following peripheral nerve injury leads to severely impaired regeneration (Werner et al., 2000; Gardiner et al., 2005). In contrast, the low levels of integrin receptors on CNS axons (Wallquist et al., 2004; Eva et al., 2012) leaves these axons without the necessary adhesion molecules to re-grow through lesion sites characterised by a dramatic up-regulation of ECM components (Jones, 1996; Eva et al., 2012). Moreover, SCI has been shown to lead to inactivation of already scarce integrins at the lesion site, adding further difficulty to the task of growing through the dense ECM (Tan et al., 2011).

Each of these intrinsic mechanisms contributes to the limited growth capacity observed in many CNS neurons after injury. This suggests that if the growth state of CNS neurons could somehow be enhanced, by application of growth factors for example, significant regeneration could be achieved (discussed in section 1.5).

Concomitant with the poor intrinsic growth capacity of CNS neurons, there is also minimal trophic support available and a large number of extrinsic inhibitory molecules present in the adult CNS following injury which further contribute to the lack of repair.

1.4.2 Lack of trophic support

When comparing injury in the CNS to injury in the PNS it becomes apparent that there is a distinct lack of support in the CNS, both physical and trophic, for any axons with the capability to regenerate. For example, PNS injury leads to Schwann cell proliferation and dedifferentiation into a non-myelinating phenotype that effectively supports axonal regeneration, whilst in the CNS oligodendrocytes fail to dedifferentiate and axons are presented with the physical barrier of a glial scar surrounded by a fluid filled cavity which provides no substrate for axons to grow on (Fenrich and Gordon, 2004). In addition to this lack of physical support, CNS axons also suffer from a significant lack of trophic support post-injury in comparison to their PNS counterparts. Injury in the PNS triggers the rapid production of several neurotrophic growth factors by Schwann cells, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-4 (NT-4) (Meyer et al., 1992; Sendtner et al., 1992; Funakoshi et al., 1993; Curtis et al., 1993; Naveilhan et al., 1997; Giger et al., 2010). By contrast, there is limited up-regulation of growth factors in the injured CNS, particularly at the injury site, and some studies even suggest that growth factor levels are decreased (Widenfalk et al., 2001; Hagebrahimi et al., 2008; Hyun et al., 2009; Giger et al., 2010). Combined with the injury-induced decrease in neurotrophin receptors previously discussed, there is clearly an insufficient level of trophic support for axons in the injured CNS.

1.4.3 Extrinsic inhibitory factors

As previously mentioned, if injured CNS axons are provided with a growth permissive substrate many of them will grow into it with varying degrees of success (Richardson et al., 1980;David and Aguayo, 1981;Cheng et al., 1996;Li et al., 1997;Houle et al., 2006), indicating that whilst many CNS neurons may have a lower intrinsic capacity than their peripheral counterparts, they are still capable of steady regenerative growth if the inhibitory environment of the CNS is replaced with a permissive PNS environment. Since the earliest indications that the local CNS environment was non-growth permissive extensive research has been carried out to identify exactly what it is in the CNS that so potently inhibits axonal growth, particularly following injury.

1.4.3.1 *Inhibitory guidance molecules*

Chemorepulsive axon guidance molecules play a vital role in the correct wiring of the CNS during development, but there are now a number of studies which suggest that they also play a role in restricting axonal growth following injury. For example, the semaphorin family are potent repellents of axon growth during development, helping to shape the developing CNS by deflecting axons from inappropriate areas (Messersmith et al., 1995;Behar et al., 1996). Semaphorin-3A is also produced by meningeal fibroblasts in the glial scar (Pasterkamp et al., 2001;Pasterkamp and Verhaagen, 2001) and enhanced regeneration of axons is observed following Semaphorin-3A inhibition (Kikuchi et al., 2003). Eph receptor tyrosine kinases and their ligands, ephrins, are also important inhibitory guidance molecules during development and several eph receptors are up-regulated following SCI (Miranda et al., 1999;Willson et al., 2002) whilst some ephrins are also upregulated at the site of injury by astrocytes (Bundesen et al., 2003). The ephrin, EPHA4, is the most studied in the context of SCI, with genetic knockout or application of a blocking peptide for this protein both resulting in an enhanced

regenerative response (Goldshmit et al., 2004; Fabes et al., 2007). EPHB3 has also been implicated in the inhibition of axonal regeneration following CNS injury, its genetic knockout resulting in enhanced regeneration of CNS axons following optic nerve crush or spinal hemisection (Duffy et al., 2012).

1.4.3.2 Myelin-associated inhibition

Berry (Berry, 1982) was the first to suggest that factors associated with CNS myelin may be inhibitory to axonal outgrowth following CNS injury, pointing out that non-myelinated CNS axons could regenerate following injury if nearby myelin remained intact, but not if myelin became damaged, and therefore hypothesising that the degeneration products of myelin were inhibitory to axonal growth. Since it was originally hypothesised that CNS myelin was inhibitory to axonal growth, three key myelin-associated inhibitory molecules have been identified: Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp). Nogo was the first of the myelin-associated inhibitors to be studied in detail. Original *in vitro* studies found that removal of two membrane protein fractions, of 35 kD and 250 kD, from CNS myelin yielded a myelin based substrate which was permissive for growth, whilst addition of these fractions to a normally growth-permissive substrate resulted in potent inhibition of neurite outgrowth (Caroni and Schwab, 1988a). An antibody raised against these inhibitory myelin fractions, IN-1, was shown to enhance neurite outgrowth both *in vitro* (Caroni and Schwab, 1988b) and *in vivo* (Schnell and Schwab, 1990). Further studies showed that the white matter of both brain and spinal cord cryosections was inhibitory to neuronal adhesion and neurite outgrowth, whilst the grey matter and PNS tissue were growth permissive. Reduction of oligodendrocytes and myelin content on these CNS cryosections reduced their inhibitory properties, as did the binding of the IN-1 antibody (Savio and Schwab, 1989). Additionally, suppression of myelin formation by

γ -irradiation in early post-natal rats was shown to result in regeneration of corticospinal axons *in vivo* following their transection 2 weeks after irradiation, despite the rats having reached a stage of development at which axonal regeneration is normally minimal (Savio and Schwab, 1990). Eventually Nogo-A was identified as the main target of the IN-1 antibody (Chen et al., 2000;GrandPre et al., 2000;Prinjha et al., 2000). Prior to the identification of Nogo-A as the target for IN-1, MAG (McKerracher et al., 1994;Mukhopadhyay et al., 1994) and subsequently OMgp (Wang et al., 2002b) were also identified as potent inhibitors of neurite outgrowth *in vitro*. However, knockout of both MAG and OMgp in transgenic mice appears to have little effect following injury *in vivo*. When combined with additional knockout of Nogo-A however, greater axonal outgrowth and functional recovery was achieved than that which was observed with knockout of Nogo-A alone, suggesting a synergistic role for these inhibitory myelin proteins (Cafferty et al., 2010).

All three of these molecules are able to bind to the same GPI-linked receptor, NgR1 (including all three CNS isoforms of Nogo: Nogo-A, -B and -C) (Fournier et al., 2001;Domeniconi et al., 2002;Liu et al., 2002a;Wang et al., 2002b). NgR1 was first identified as the receptor for a 66 amino acid loop conserved between Nogo isoforms, Nogo-66, which was known to confer many of the inhibitory effects of Nogo (Fournier et al., 2001). NgR1 forms a signalling receptor complex with LINGO-1 (Mi et al., 2004) and either p75 (Wong et al., 2002;Wang et al., 2002a) or TROY (Park et al., 2005;Shao et al., 2005) as it has no transmembrane domain so requires association with membrane proteins to induce signalling (Schwab, 2010). Activation of this complex is thought to lead to downstream signalling via the activation of the small GTPase RhoA and its effector protein Rho kinase (ROCK), ultimately resulting in growth cone collapse (Lehmann et al., 1999;Niederost et al., 2002;Schwab, 2010). More recently, paired

immunoglobulin-like receptor B (PirB) has been identified as a second receptor for Nogo-66, MAG and OMgp (Atwal et al., 2008). Whilst there is clear evidence that these myelin-associated proteins have a strong inhibitory effect on axonal outgrowth *in vitro*, there is continued debate on the extent to which they contribute to the failed regenerative response of the CNS *in vivo*; results achieved by genetic ablation of Nogo in particular are conflicting and variable, with a number of studies suggesting that the effects of Nogo knockout are minimal (Zheng et al., 2003; Woolf, 2003; Zheng et al., 2005; Steward et al., 2008; Lee et al., 2009; Silver, 2010; Lee et al., 2010b; Lee et al., 2010c) and others indicating that expression of axonal NgR1 is low in the spinal cord of both rodents and humans and remains so after injury (this will be discussed in greater detail in section 1.5.5) (Hunt et al., 2002; Josephson et al., 2002).

1.4.3.3 Proteoglycans

The glial scar that forms at the lesion site post-SCI presents not only a physical barrier to regeneration, but the cells of the glial scar (reactive astrocytes in particular) also produce a number of inhibitory ECM molecules, creating a chemical barrier to regeneration (Reier et al., 1983; Silver and Miller, 2004; Fitch and Silver, 2008; Sharma et al., 2012). In the context of SCI the most widely studied of these up-regulated inhibitory molecules are the proteoglycans. Proteoglycans consist of a core protein with covalently attached glycosaminoglycan (GAG) side chains and are classified according to the nature of these side chains, which can be chondroitin sulphate (CS), dermatan sulphate, keratan sulphate or heparan sulphate GAGs. Whilst there is evidence to suggest that keratan sulphate proteoglycans (KSPGs), heparan sulphate proteoglycans (HSPGs) and CSPGs are all up-regulated following injury to the CNS (Fitch and Silver, 1997; Lemons et al., 1999; Jones and Tuszynski, 2002; Jones et al., 2003b; Jones et al., 2003c; Ramer et al., 2005; Ito et al., 2010), CSPGs in particular and their effects in the

injured CNS have been the focus of extensive research efforts (Fitch and Silver, 1997; Galtrey and Fawcett, 2007; Fitch and Silver, 2008; Bradbury and Carter, 2010; Bartus et al., 2012). CS-GAGs are made up of repeating disaccharide units of glucuronic acid and N-acetyl galactosamine (Kjellen and Lindahl, 1991; Sugahara et al., 2003), with a varying number of CS-GAG side chains on differing members of the CSPG family (Figure 1.3.). These sugar residues can be mono- or disulphated, resulting in highly variable patterns of sulphation which determine the interactions of CS-GAGs with other molecules (Gama et al., 2006; Sugahara and Mikami, 2007). The lectican family of CSPGs (neurocan, brevican, aggrecan and versican – Figure 1.3) are the most abundant in the CNS and can be secreted by the cells of the glial scar, creating a CSPG rich ECM around the injury site (Asher et al., 2000; Asher et al., 2002; Jones et al., 2003b) whilst other CSPGs, such as NG2, are expressed on glial cell membranes (Dou and Levine, 1994; Levine, 1994). In rodent models of SCI, glial scar CSPGs are rapidly upregulated, with CSPGs such as NG2 and neurocan dramatically increased as early as 24 hours post-injury and peaking 8-14 days later. Although some CSPGs are actually reduced following injury, such as brevican which is significantly reduced for 14 days post-injury before beginning to return to normal levels by 28 days (Andrews et al., 2012). While the post-injury time course of expression for different CSPGs may vary, overall CSPG expression remains dramatically increased well into the chronic post-injury stages (Lemons et al., 1999; Tang et al., 2003; Jones et al., 2003b; Iaci et al., 2007; Andrews et al., 2012).

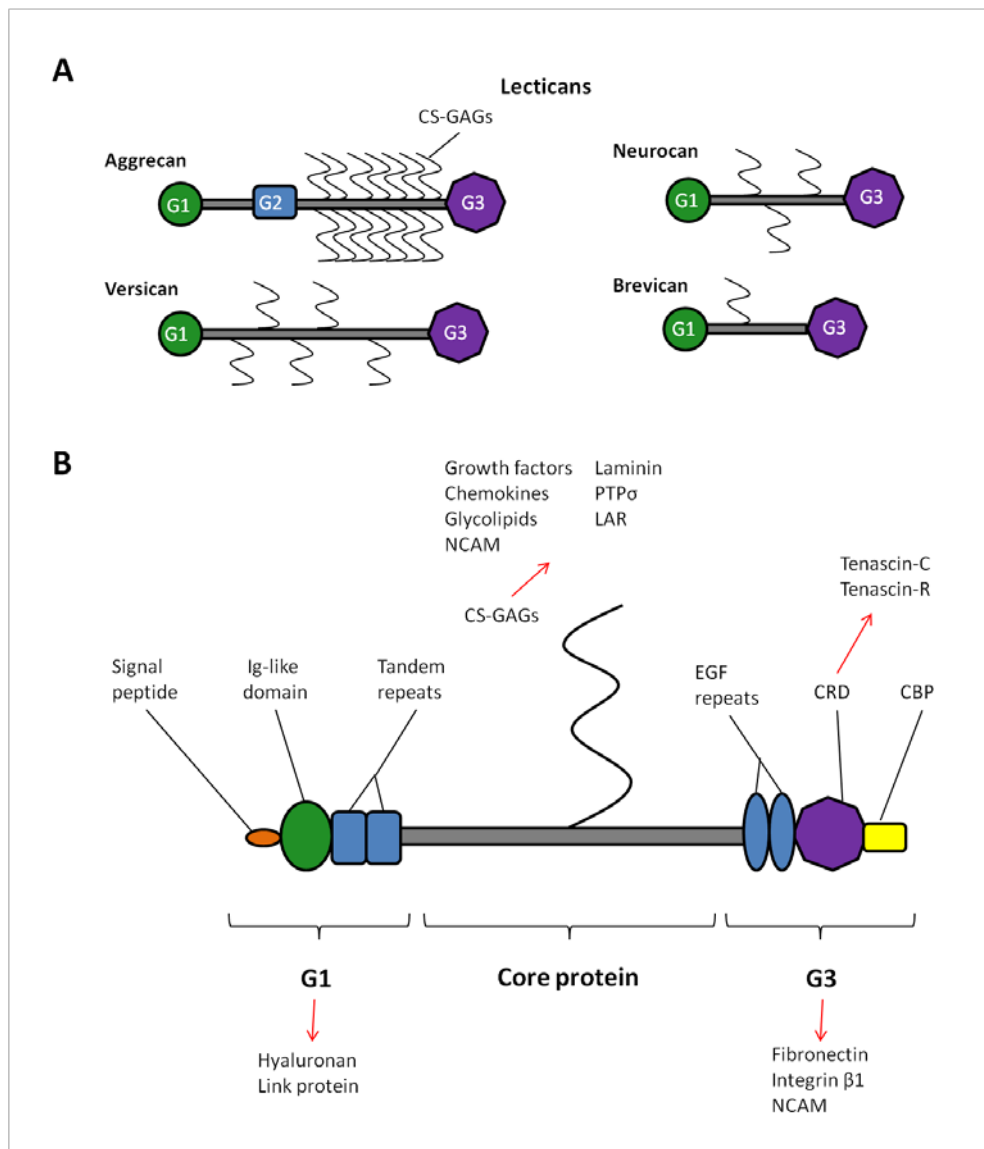


Figure 1.3. Schematic representation of some of the most common CNS CSPGs (the lecticans) and their typical environmental interactions. (A) Lecticans are composed of two globular N- and C-terminal domains (G1 and G3, respectively) flanking a core protein region where CS-GAGs attach via serine residues. Aggrecan has an additional G2 domain adjacent to the G1 domain. (B) Schematic showing molecular components of the G1 and G3 domains of a lectican, indicating target areas for interactions with binding partners and interactions with other ECM molecules (red arrows). CRD, Carbohydrate recognition domain; CBP, complement binding protein; EGF, epidermal growth factor; Ig, immunoglobulin.

The inhibitory role of CSPGs on axon growth has been demonstrated in many ways; *in vitro* studies have shown that sensory neurons fail to extend neurites on a normally growth permissive substrate if it contains CSPGs (Snow et al., 1990), that adult CNS neurons fail to extend neurites on glial scar explants (Rudge and Silver, 1990;McKeon et al., 1991;McKeon et al., 1995), that contact with CSPGs disrupts growth cone dynamics (Snow et al., 1996) and that NG2 is one of the most inhibitory CSPGs and is produced by astrocytes and oligodendrocyte/type 2 astrocyte progenitors which react rapidly to CNS injury (Fidler et al., 1999). *In vivo* evidence has shown that transplanted adult sensory neurons will extend axons in the CNS, but that these axons will halt abruptly and form dystrophic end bulbs upon reaching a site of reactive gliosis, correlating with areas of increased CSPG expression (Davies et al., 1997;Davies et al., 1999). Since these early studies highlighting the link between CSPGs in the glial scar and inhibition of axonal growth, CSPG-mediated inhibition has been identified as a key mechanism underlying the lack of a regenerative response in the injured CNS. This is due to numerous studies showing that disruption of CSPGs and their inhibitory interactions, whether it is via enzymatic degradation, inhibition of synthesis, anti-body neutralisation or by targeting downstream effector molecules, dramatically improves axonal growth both *in vivo* and *in vitro* (McKeon et al., 1995;Moon et al., 2001;Bradbury et al., 2002;Grimpe and Silver, 2004;Grimpe et al., 2005;Shen et al., 2009;Fry et al., 2010;Fisher et al., 2011;Brown et al., 2012).

Despite CSPGs having been revealed as an inhibitor of axonal growth nearly 25 years ago, it is only recently that a better understanding has been gained of some of the mechanisms through which CSPG-mediated inhibition is achieved. CSPGs are thought to inhibit axonal growth via a variety of mechanisms, both direct and indirect. Many of

the indirect mechanisms of CSPG-mediated inhibition have been known for a long time. For example, integrins are important regulators of neuronal growth and CSPGs have been shown to inhibit regeneration via inactivation of, and competitive binding to, integrins (Condic, 2001;Zhou et al., 2006;Tan et al., 2011). Furthermore, CSPGs are known to bind to growth permissive substrates, such as laminin and neural cell adhesion molecule (NCAM), in the CNS (Zuo et al., 1998a;Sasaki et al., 2001), thereby preventing access to axons attempting to grow on these substrates. CS-GAGs have also been shown to bind to various growth factors and thus competitive binding again prevents access to molecules beneficial to a regenerative response (Deepa et al., 2002;Nandini et al., 2004;Nandini and Sugahara, 2006). The discovery of direct mechanisms through which CSPGs mediate their inhibitory effects have only been made recently. Two closely related functional receptors for CSPGs have been identified, protein tyrosine phosphatase σ (PTP σ) (Shen et al., 2009;Fry et al., 2010) and leukocyte common antigen-related phosphatase (LAR) (Fisher et al., 2011). The Nogo receptors NgR1 and NgR3 have also been proposed as receptors for CSPGs (Dickendesher et al., 2012). Whilst the downstream signalling pathways controlled by these receptors are complex and not completely understood, it has been proposed that all of these receptors can inhibit axon growth and induce growth cone collapse via activation of the RhoA/ROCK signalling pathway (McGee and Strittmatter, 2003;Fisher et al., 2011;Sharma et al., 2012). The vast majority of these interactions are mediated via binding sites on the CS-GAG side chains identified by specific sulphation patterns. The dramatic and sustained upregulation of CSPGs following injury, combined with their multi-faceted inhibitory actions mean that these molecules remain a key target for the development of potential therapeutic interventions for use in SCI (discussed in section 1.5.7).

1.5 Strategies to promote repair

There is clearly a significant need to address the lack of repair following spinal SCI and intensive research has focussed on improving our understanding of the complex processes involved in SCI as well as developing experimental therapeutics to target some of these processes, with the ultimate goal of improving neurological outcomes and quality of life for patients. Great progress has been made in working towards this goal over the last 20 years, such that there is genuine belief within the field that we will one day be able to significantly reduce the debilitating consequences of SCI. As outlined in the previous sections, there appear to be four main potential strategies for treating SCI: preventing, or reducing, the processes that lead to extensive secondary damage of the spinal cord; enhancing the intrinsic growth potential of CNS neurons; removing, or reducing, the intensely inhibitory properties of the injured CNS environment; enhancing endogenous repair mechanisms such as plasticity and strengthening of compensatory pathways. These four strategies are by no means mutually exclusive and targeting two or more of these areas, whether with a single therapeutic or using a combinatorial approach, is likely to lead to more significant effects than addressing just one. The development of experimental strategies designed to address these issues will be discussed in the sections below and are summarised in figure 1.4.

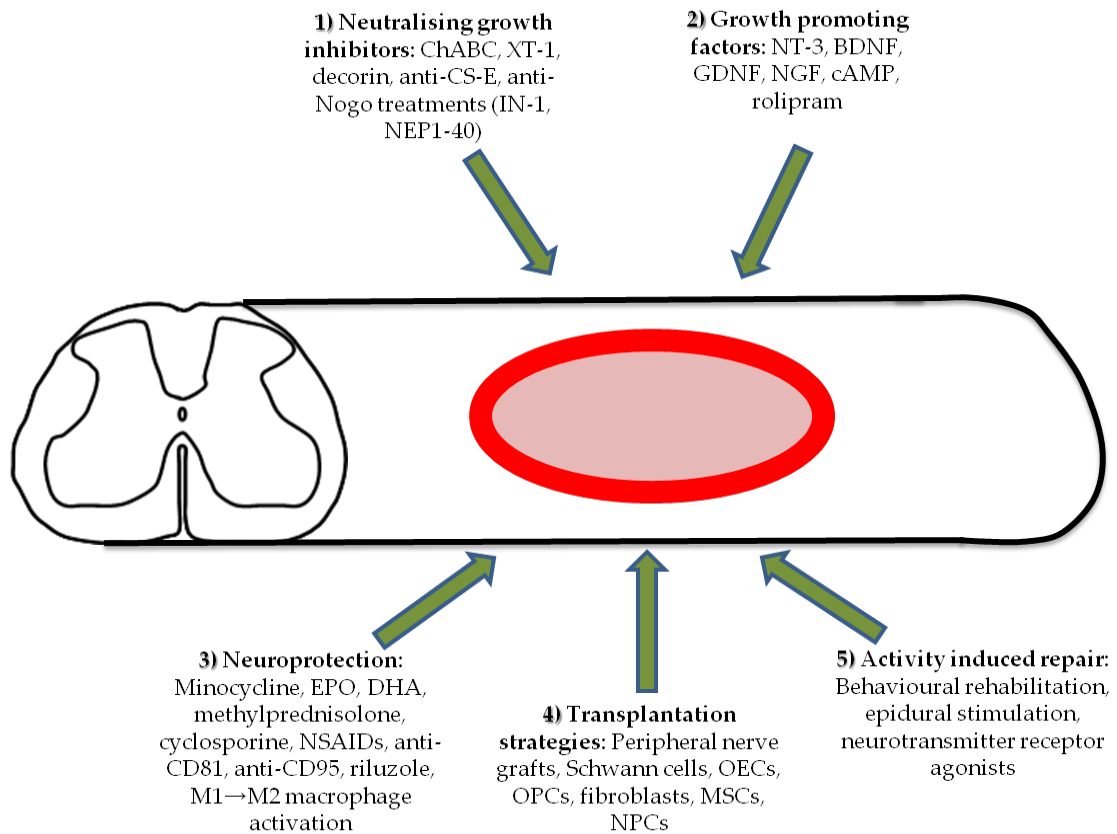


Figure 1.4. Schematic summarising the various strategies to promote repair discussed in section 1.5. A wide array of strategies to promote functional recovery are being pursued in SCI research. This has resulted in the development of a substantial number of experimental therapeutics and interventions targeting varying aspects of repair, many of which are discussed in section 1.5 and these are summarised in the above schematic. ChABC, chondroitinase ABC; XT-1, xylotransferase-1; NT-3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; NGF, nerve growth factor; EPO, erythropoietin; DHA, docosahexaenoic acid; OECs, olfactory ensheathing cells; OPCs, oligodendrocyte precursor cells; MSCs, multipotent stromal cells; NPCs, neural progenitor cells.

1.5.1 Neuroprotection

As discussed previously, the inflammatory response to SCI plays a prominent role in the secondary damage that occurs post-injury. For this reason a number of therapeutic strategies have aimed to modulate or inhibit the inflammatory response in an attempt to attenuate secondary damage. Anti-inflammatory and immunosuppressant drugs have therefore been used in rodent models of SCI to varying effects, including minocycline (Stirling et al., 2004;Pinzon et al., 2008), non-steroidal anti-inflammatory drugs (NSAIDs) (Simpson, Jr. et al., 1991;Harada et al., 2006) and cyclosporine (Rabchevsky et al., 2001). Whilst some studies have shown beneficial effects of anti-inflammatory drugs on tissue sparing and functional recovery (Simpson, Jr. et al., 1991;Stirling et al., 2004), others have found no effects (Rabchevsky et al., 2001;Pinzon et al., 2008) or even detrimental effects (Harada et al., 2006). To add to this confusion, genetic ablation of reactive astrocytes has been shown to lead to prolonged failure of the blood-spinal cord barrier, prolonged and widespread inflammation, increased tissue damage and ultimately, decreased functional recovery (Bush et al., 1999;Faulkner et al., 2004), yet reduction in astrocyte signalling and proliferation via inhibition of CD81 can lead to decreased secondary damage and enhanced functional recovery (Dijkstra et al., 2006). A similar story is apparent when interfering with neutrophil infiltration, with both positive (Gris et al., 2004;Geremia et al., 2012) and negative (Stirling et al., 2009) effects reported. The inflammatory response to SCI is particularly complex and known to have both detrimental and beneficial effects, perhaps explaining why strategies to suppress the immune response can lead to such contrasting results. Recently a number of studies have focussed on modulating the immune response to promote an immune phenotype that is more beneficial than detrimental (Ghasemlou et al., 2010a;Ghasemlou et al., 2010b;Donnelly et al., 2011). Macrophages in particular appear to be a prime target for

immunomodulation by encouraging a change in phenotype from pro-inflammatory M1 activation to a reparatory M2 activation (Kigerl et al., 2009;Ghasemlou et al., 2010b;David and Kroner, 2011;Donnelly et al., 2011). Whilst targeting the immune response can undoubtedly have beneficial effects, timing and targeting of immune-altering therapeutics will be vital and requires careful consideration due to the complexities and time course of the SCI immune response.

Although modulating inflammation has been a prominent target for neuroprotective strategies, a number of other targets also exist for promoting neuroprotection. For example, glutamate excitotoxicity can be reduced by using magnesium to block NMDA receptors (Ditor et al., 2007;Wiseman et al., 2009) or by blockade of tetrodotoxin-sensitive sodium channels using riluzole, resulting in improved ionic homeostasis and reduced glutamate release (Stutzmann et al., 1996;Schwartz and Fehlings, 2001). Pro-apoptotic pathways can also be targeted. For example, both erythropoietin (Brines et al., 2004;Okutan et al., 2007) and inhibition of CD95 signalling (Demjen et al., 2004) result in decreased apoptosis due to disruption of apoptotic program induction. Finally, omega-3 polyunsaturated fatty acids (such as docosahexaenoic acid) have been shown to have neuroprotective effects in both neurodegenerative diseases as well as traumatic injuries (Dyall and Michael-Titus, 2008;Ward et al., 2010;Figuerola et al., 2012); the mechanisms through which this neuroprotection is mediated are not well understood however. Prevention or reduction of secondary damage can lead to improved functional outcomes, but in order for this to be the case treatment must be administered as soon as possible to optimise reduction of secondary pathology and this may not always be a realistic possibility. Encouraging repair of the injured spinal cord may, therefore, represent a more robust therapeutic strategy which would be more applicable to both acute and chronic SCI.

1.5.2 Priming neurons for regeneration

As previously discussed, the regenerative response in the PNS is far more effective than that of the CNS and this has prompted research into the effect of a PNS lesion on CNS regeneration in neurons that have both a peripheral and a central axon – dorsal root ganglia (DRG) neurons. Initial studies showed that transection of the peripheral axons of DRG neurons prior to transection of their central axons resulted in a dramatic increase in the number of their axotomised central axons that would grow into a peripheral nerve graft (Richardson and Issa, 1984). These results have been replicated a number of times and it has additionally been shown that, after axotomy of peripheral DRG axons, significant regeneration of central DRG axons can be seen into and beyond a CNS lesion even without a peripheral nerve graft (Chong et al., 1996;Neumann and Woolf, 1999). The effects of peripheral axotomy on central regeneration were optimal if axotomy of peripheral branches occurred a week prior to axotomy of central branches (Richardson and Issa, 1984;Neumann and Woolf, 1999), suggesting that a peripheral “conditioning” injury led to cellular changes that boosted the regenerative capabilities of DRG neurons. This technique therefore became known as a conditioning lesion. Later studies revealed that upregulation of RAGs and elevated levels of cyclic adenosine monophosphate (cAMP) in the neuronal cell bodies of DRG neurons were key mechanisms underlying this conditioning effect (Schreyer and Skene, 1993;Bradbury et al., 2000;Bulsara et al., 2002;Qiu et al., 2002a;Qiu et al., 2002b;Ramer et al., 2005). A number of therapeutic strategies have therefore targeted elevating cAMP levels in order to replicate the effects of a conditioning lesion. Cell permeable dibutyryl cAMP has been administered directly to DRGs prior to injury of dorsal column axons, resulting in similar effects to those observed following a conditioning lesion (Neumann et al., 2002;Qiu et al., 2002a). Rolipram is an inhibitor of

phosphodiesterase, the enzyme responsible for cAMP degradation, and its subcutaneous administration has been shown to result in elevated cAMP levels and mimic the effects of a conditioning lesion (Pearse et al., 2004; Nikulina et al., 2004). However, a more recent study has shown that whilst strategies which elevate cAMP levels recapitulate many of the effects of a conditioning lesion, they do not entirely replicate them. Conditioning lesions led to a more substantial and more sustained upregulation of transcriptional regulators and RAGs than elevated cAMP alone, ultimately resulting in a more pronounced effect on sensory axon regeneration following cervical dorsal column transection (Blesch et al., 2012b). The fact that delivery of these “conditioning” treatments is optimal prior to injury limits their usefulness in clinical terms. However, conditioning strategies do exert beneficial effects if given at the time of injury in combination with other therapeutics, particularly those involving the provision of a more growth permissive substrate to CNS axons (such as cellular or peripheral nerve grafts) (Lu et al., 2004; Pearse et al., 2004; Nikulina et al., 2004). Even as part of a combination treatment however, the additive effect of a conditioning strategy is greatly diminished when administered at the time of injury, or soon after, in comparison to pre-injury conditioning (Wang et al., 2012)

1.5.3 Neurotrophic factors

Another way in which the intrinsic regenerative capacity of CNS neurons can be boosted is via the application of various neurotrophic factors. Neurotrophic factors are key modulators of neuronal survival, growth, plasticity and neurotransmission and have therefore been delivered to the spinal cord through a variety of mechanisms in attempts to promote neuronal growth, repair and survival following SCI (Plunet et al., 2002; Lu and Tuszynski, 2008; Hollis and Tuszynski, 2011). For example, local infusion of NT-3, BDNF or GDNF have all been shown to promote axonal growth and/or neuronal

survival following CNS injury (Giehl and Tetzlaff, 1996;Bradbury et al., 1998;Giehl et al., 1998;Bradbury et al., 1999;Kwon et al., 2002). Differing neurotrophins appear to affect individual neuronal populations differently; for instance, NT-3 has been shown to promote growth of dorsal column sensory axons and CST axons (Schnell et al., 1994;Bradbury et al., 1999) whilst BDNF has been shown to promote sprouting of rubrospinal and raphespinal projections (Kobayashi et al., 1997;Ye and Houle, 1997) but to have little effect on ascending sensory projections (Bradbury et al., 1998;Bradbury et al., 1999).

The use of genetically modified cellular grafts is another regularly employed technique for delivery of neurotrophins (Liu et al., 2002b;Tobias et al., 2003;Tuszynski et al., 2003;Blesch et al., 2004;Lu et al., 2005). The presence of a cellular graft provides a growth-permissive substrate for axons to grow within, whilst the presence of neurotrophins boosts the intrinsic regenerative capacity of the axons. However, the strong concentration gradient of growth promoting neurotrophins being secreted from cellular grafts often results in axons growing towards and into the graft, but not beyond it (Tobias et al., 2003;Blesch et al., 2004;Lu et al., 2005). To overcome this problem, Alto and colleagues (2009) used a cellular graft at the site of injury and then injected a lentiviral vector (LV) expressing NT-3 into either an appropriate or inappropriate brainstem target for the regenerating axons. Axons regenerated through the graft at the lesion site and on towards the cells that were producing NT-3, whether they were an appropriate target or not, where they appeared to form synapses, although these connections were not functional (Alto et al., 2009). This study highlights that neurotrophin gradients can be used to direct regenerating axons towards specific targets, although this task clearly becomes increasingly complex when numerous spinal pathways are disrupted. Whilst the administration of exogenous neurotrophic factors

clearly boosts the intrinsic growth capacity of CNS neurons, issues can arise from the fact that axons will often grow towards a neurotrophin gradient, but not beyond it, and can potentially form inappropriate connections. In addition, the effect of some neurotrophins (e.g. NGF and GDNF) on promoting growth of nociceptive fibres can lead to increased pain states (Romero et al., 2000; Ramer et al., 2003). Neurotrophins therefore provide a promising repair strategy, particularly as part of a combination treatment, but their use will require careful manipulation to ensure they are used effectively. For instance, the over-expression of NGF in the dorsal horn of the spinal cord results in regeneration of small-diameter afferent fibres which form appropriate functional connections in the superficial dorsal horn, as well inappropriate connections in the deep dorsal horn which causes severe hyperalgesia (Tang et al., 2004). The co-expression of NGF with the chemorepulsive guidance molecule semaphorin 3A in the ventral horn however, prevents this overshoot and inhibits the development of hyperalgesia (Tang et al., 2004). It may therefore be possible to carefully guide axonal regeneration towards appropriate targets using neurotrophins to boost growth and chemorepulsive gradients to prevent growth into inappropriate areas. The disruption of numerous spinal pathways following SCI however, means that carefully directed regeneration of multiple pathways would be overly complex and likely unachievable.

1.5.4 Transplantation of growth-permissive substrates

The transplantation of growth permissive, and often growth promoting, substrates into a site of spinal injury can be used to target a number of issues associated with SCI. Not only can grafts, whether cellular or tissue based, provide a bridging substrate for gaps created by tissue damage and provide a permissive conduit for CNS axons to grow into, they can also be genetically modified to secrete factors which actively promote growth or are targeted at neutralising the inhibitory environment of the injured spinal cord.

Whilst the use of a peripheral nerve graft gave the first indication that CNS axons could regenerate into a permissive environment and even bypass a lesion (Richardson et al., 1980;David and Aguayo, 1981), the use of specific cell-type transplants has become a more commonly used strategy in pre-clinical research for a number of reasons: single cell types can be characterised *in vitro* and are better defined than the assortment of cells present in a nerve graft; they can be injected as a suspension to fill a lesion site whereas a nerve graft requires implantation that is likely to cause further damage; and cell suspensions can be genetically modified more easily to secrete factors such as neurotrophins (Ramer et al., 2005). A wide variety of cell types have been used to provide a bridging substrate in the damaged spinal cord and provide a growth-permissive environment. These include: olfactory ensheathing cells (OECs) (Li et al., 1997;Ramon-Cueto et al., 2000;Toft et al., 2007), Schwann cells (Xu et al., 1995;Bunge, 2002), multipotent stromal cells (MSCs) (Hofstetter et al., 2002;Sasaki et al., 2009), oligodendrocyte precursor cells (OPCs) (Keirstead et al., 2005;Cao et al., 2010), neural progenitor cells (NPCs) (Karimi-Abdolrezaee et al., 2006;Bonner et al., 2011) and fibroblasts (Tuszynski et al., 1994;Tuszynski et al., 1997). The different cell types that have been transplanted into the injured cord all have various advantages and disadvantages when compared with one another and there is therefore debate as to which cell type provides the most viable therapeutic option. These will be discussed in more detail below.

1.5.4.1 Multipotent stromal cells and neural progenitor cells

Both MSCs and NPCs can be harvested from adult tissue and are often loosely termed as stem cells due to their multipotent nature (Ruff et al., 2012). Injured CNS axons grow into grafts of either cell type (Hofstetter et al., 2002;Lu et al., 2003;Ankeny et al., 2004), indicating that both cell types can function as a permissive bridging substrate. The

multipotency of these cells also raises the possibility that differentiation could be induced to replace lost oligodendrocytes or spinal circuitry. Although differentiation of MSCs into neural cells is possible *in vitro*, it is unclear whether this occurs to any meaningful extent *in vivo* (Castro et al., 2002; Vallieres and Sawchenko, 2003; Mezey et al., 2003). Whilst NPCs have been shown to be capable of differentiation into neural cells *in vivo* (Ogawa et al., 2002), in general when either of these multipotent cell types are transplanted into an injury the vast majority become glial cells if they differentiate at all (Vallieres and Sawchenko, 2003; Karimi-Abdolrezaee et al., 2006; Karimi-Abdolrezaee et al., 2010). Thus these cell types are often used as bridging substrates rather than cell replacement therapies and as such have both been shown to encourage regeneration and functional recovery, particularly when used as part of a combination strategy. For example, transplantation of MSCs at the site of a cervical dorsal column injury combined with a rostral NT-3 concentration gradient was shown to promote significant axonal regeneration beyond the MSC graft when administered at acute (Alto et al., 2009) or chronic (Kadoya et al., 2009) post-injury time points. Additionally, BDNF-secreting MSC grafts enhanced functional recovery and promoted sprouting of CST and serotonergic fibres following a thoracic hemisection (Sasaki et al., 2009). As a cell replacement strategy, NPCs have been shown to be capable of becoming oligodendrocytes and carrying out remyelination when combined with a number of growth factors and anti-inflammatory drugs (Karimi-Abdolrezaee et al., 2006). The lack of functional repair observed if these cell types are given without numerous other factors however, indicates their limitations (Sasaki et al., 2009; Karimi-Abdolrezaee et al., 2010). Additionally the relative scarcity of NPCs in the mammalian CNS, as well as the risks associated with their harvest, limit their viability as a clinical therapeutic strategy (Ruff et al., 2012). The apparent lack of meaningful differentiation of MSCs

nullifies one of their major potential advantages in that they seem unlikely to be useful as a cell replacement strategy. These cell types therefore, have many promising characteristics for treating SCI, but as yet have not achieved their full potential when used *in vivo*.

1.5.4.2 Schwann cells and olfactory ensheathing cells

As is the case with most cell types used in transplantation strategies for SCI, both Schwann cells and OECs provide structural and trophic support for regenerating axons in the injured CNS (Bunge and Pearse, 2003; Oudega and Xu, 2006; Oudega, 2007; Barnett and Riddell, 2007). An important additional benefit of these cells is that autologous cell grafts of either cell type can be easily harvested in a minimally invasive way (Bunge and Pearse, 2003; Lindsay et al., 2010). Early studies using Schwann cell transplantation showed that Schwann cell seeded guidance channels could support extensive regeneration of both ascending and descending axons following complete transection of the spinal cord (Xu et al., 1995; Xu et al., 1997). Early studies using OECs also indicated their ability to support extensive regeneration, stimulating regeneration of sensory axons across the inhibitory barrier of the dorsal root entry zone following dorsal root rhizotomy and anastomosis of the stump to the spinal cord (Ramon-Cueto and Nieto-Sampedro, 1994). The first evidence that OECs could promote significant regeneration following SCI was provided by Li *et al* (1997). In this study OECs were injected into the injury site following unilateral lesioning of the CST in the upper cervical spinal cord. BDA-traced lesioned axons were observed to regenerate through the OEC graft and reintegrate into the host CST caudal to the injury within 10 days of transplantation. Furthermore, this regeneration was associated with improved skilled reaching in the affected forepaw (Li et al., 1997). A further study showed that combination of a Schwann cell seeded guidance channel and OECs to treat a complete

spinal transection resulted in dramatic long-distance axonal regeneration through and around the guidance channel, indicating that the presence of OECs significantly enhanced the level of regeneration achieved by Schwann cells alone (Ramon-Cueto et al., 1998).

In addition to promoting regeneration, Schwann cells and OECs have the additional benefit of being able to promote myelination (Duncan et al., 1981; Devon and Doucette, 1992; Imaizumi et al., 1998; Kato et al., 2000) and thereby alleviate conduction block in focally demyelinated fibres (Imaizumi et al., 1998). Interestingly, remyelination induced by OEC transplantation may actually be carried out by endogenous Schwann cells (Boyd et al., 2004). OECs have the additional benefit that, in comparison to Schwann cells, they induce less reactive gliosis and CSPG upregulation when transplanted and migrate into host tissue (Lakatos et al., 2003; Ramer et al., 2004; Zhang et al., 2011), axons therefore have a better chance of regenerating beyond the graft. Indeed, the administration of OECs alone has been shown to promote regeneration of some spinal pathways beyond the graft and enhance functional recovery in a number of pre-clinical studies (Li et al., 1997; Ramon-Cueto et al., 2000; Lu et al., 2002; Li et al., 2003; Toft et al., 2007) as well as in a veterinary clinical trial (Granger et al., 2012). OECs are therefore, perhaps the most promising cell type for graft-based therapies in terms of clinical translation. Although functional benefits are observed when OECs are given alone, it is likely that the use of OECs as part of a combination treatment will lead to even more promising results. For example OECs could be combined with neurotrophic factors to enhance regeneration of the spinal pathways that do not normally grow beyond the OEC grafts as well as potentially guiding regeneration towards appropriate targets. Equally, the degradation of inhibitory CSPGs in the glial scar may encourage enhanced regeneration of axons beyond the site of OEC

transplantation and migration. Indeed, the full potential of any cell-based transplantation strategy is likely to depend upon the success with which it can be combined with other, synergistic therapies to achieve the restoration of functionally useful connections (Barnett and Riddell, 2007).

1.5.5 Targeting myelin-associated inhibition

Due to the potent effects of myelin-associated inhibition on axonal outgrowth, extensive research efforts have focused on developing therapeutic strategies aiming to neutralise the inhibitory effects of myelin proteins. Whilst Nogo, MAG and OMgp have all been shown to confer the inhibitory effects of myelin, Nogo has received particular attention due to early studies involving the use of the IN-1 monoclonal antibody which targets Nogo-A inhibition (see section 1.4.3.2). As well as enhancing axonal outgrowth both *in vitro* (Caroni and Schwab, 1988b) and *in vivo* (Schnell and Schwab, 1990), IN-1 also has also been shown to significantly enhance functional recovery in both rodents and primates following hemisection injury (Bregman et al., 1995; Freund et al., 2006). As well as promoting regeneration of injured axons, IN-1 has also been shown to promote plasticity of spared pathways following discrete lesions of specific spinal tracts such as the CST (Thallmair et al., 1998; Raineteau et al., 2001; Raineteau et al., 2002). A clinical trial using a humanised version of the IN-1 antibody is currently underway (SCI clinical trials will be discussed in section 5.3.2). Whilst Nogo-A is the primary target of the IN-1 antibody, other molecules associated with myelin inhibition have been targeted in different ways. For example, NEP1-40 is a peptide derived from the inhibitory Nogo-66 amino acid sequence, designed as an antagonist for NgR1. Following either intrathecal (GrandPre et al., 2002) or systemic (Li and Strittmatter, 2003) delivery it was shown to promote regeneration and sprouting in a number of spinal pathways following a hemisection injury, even if delivery was delayed (Li and Strittmatter, 2003). These

effects were associated with improved functional recovery, but could not be replicated when the study was repeated by another research group (Steward et al., 2008).

The use of transgenic mice to study myelin-associated inhibition has also led to inconsistent findings, resulting in an unclear picture with regard to the contribution of these proteins to regenerative failure in the CNS. For example, in one transgenic line of Nogo-A knockout mice, only modest improvements in axonal regeneration were observed *in vivo* following spinal hemisection, whilst functional data was not reported (Simonen et al., 2003). A strong upregulation of Nogo-B may have contributed to the modesty of these findings. Furthermore, a transgenic mouse line lacking both Nogo-A and B was initially reported to display robust CST regeneration and functional recovery following hemisection (Kim et al., 2003). However, the anatomical findings of this study were later suggested to be at least partially attributable to tracer artefact (Cafferty et al., 2007; Steward et al., 2007) and a second study using the same mouse line found there to be no anatomical or functional improvements in these mutant mice (Lee et al., 2009). Mutation of all three CNS Nogo isoforms in two separate transgenic lines also appears to have minimal effects on regeneration (Zheng et al., 2003; Lee et al., 2009). Genetic knockout of NgR1 has also failed to produce consistent results, with modest anatomical and functional improvements observed in two studies (Kim et al., 2004; Harel et al., 2010) and no *in vitro* or *in vivo* effect observed in a third (Zheng et al., 2005). Whilst the inconsistency of these results seems somewhat surprising in light of the fact that Nogo, MAG and OMgp all signal through NgR1, compensatory activity of the more recently discovered PirB may contribute to the lower levels of regeneration than originally expected.

In all of the Nogo transgenic mouse lines, myelin from mutant mice was shown to be significantly less inhibitory to neurite outgrowth *in vitro* (Kim et al.,

2003;Simonen et al., 2003;Zheng et al., 2003;Lee et al., 2009), emphasising that, at least *in vitro*, Nogo is a potent growth inhibitor. The inconsistency of the *in vivo* results however, has led to uncertainty over the physiological significance of this in murine models of SCI. Interestingly, the myelin from mice deficient for MAG and OMgp was shown to be no less inhibitory than wild-type myelin *in vitro* and a similar picture was observed *in vivo*, with mutant mice responding to injury no differently than wild-types (Cafferty et al., 2010). However, the intercrossing of these mice with previously studied Nogo-A/B deficient mice (Kim et al., 2003) resulted in mice lacking MAG, OMgp and Nogo-A/B, which appeared to have a more robust regenerative response following injury than mice lacking only Nogo-A/B (Cafferty et al., 2010). This suggests that perhaps other myelin-associated inhibitors, such as MAG and OMgp, may play a compensatory role in the absence of Nogo and could contribute to the variability of results observed in Nogo transgenic mouse lines. Overall however, the disappointing and inconsistent effects of genetic ablation of the various myelin-associated inhibitors suggests that whilst CNS myelin is a potent inhibitor of axon growth *in vitro*, its contribution to the failed regenerative response post-SCI is likely to be less than originally proposed.

1.5.6 Neurorehabilitation

Exercise and neurorehabilitation is currently the primary therapy used for SCI in the clinical setting and is widely accepted to have functional benefits for patients (Peckham and Knutson, 2005;Edgerton et al., 2006;Thuret et al., 2006;Dietz, 2009). Neurorehabilitation in the clinic can take many forms including physiotherapy, weight-supported treadmill training and/or therapeutic electrical stimulation. Other than its ability to ameliorate atrophy of skeletal muscle, which otherwise can develop quickly due to a lack of weight loading and normal movement, rehabilitation can lead to

improved function, such as stepping ability, in patients (Peckham and Knutson, 2005; Hicks et al., 2005; Dietz, 2009). It is thought that regular activation of spared connections during rehabilitation helps to strengthen existing connections and maximise remaining function. In the pre-clinical setting animal models are used to aid our understanding of beneficial changes resulting from neurorehabilitation (Ying et al., 2005; Courtine et al., 2009; Ichiyama et al., 2008; Lavrov et al., 2008; Cote et al., 2011), as well to develop novel multi-component neurorehabilitative strategies aimed at further enhancing the potential for functional recovery (Hutchinson et al., 2004; Courtine et al., 2009; Chen et al., 2011; van den Brand et al., 2012; Dominici et al., 2012). Improvements occur because spinal circuitry below the lesion site remains intact and can respond to peripheral input, thereby keeping spinal circuitry active and capable of initiating central pattern generator (CPG) activity. Recurring activation of this circuitry can result in endogenous production of neurotrophins (Ying et al., 2005; Cote et al., 2011), modulation of multiple neurotransmitter systems and significant plasticity of spinal circuitry (Edgerton et al., 2004; Edgerton et al., 2008), even resulting in the formation of compensatory relay networks that can restore supraspinal control (Courtine et al., 2009).

In terms of aiding in the development of novel neurorehabilitative strategies, pre-clinical studies have employed a number of different approaches in order to regularly activate spared fibres with the aim of strengthening existing connections and potentially stimulating the formation of new ones. Classically this has involved repetitive physical training, such as regular repetition of a specific task e.g. skilled reaching for a food reward. For example, task-specific behavioural rehabilitation using a single pellet retrieval task, resulted in significantly improved performance in this task following a cervical dorsolateral quadrant lesion in comparison to rats receiving no rehabilitation (Girgis et al., 2007). Interestingly this task-specific rehabilitation resulted

in detrimental effects on performance in a different behavioural task, namely the horizontal ladder. Plasticity of the CST was associated with rehabilitative training and task-specific improvements in this study (Girgis et al., 2007). Another recent study also showed functional recovery following task-specific rehabilitation, using horizontal ladder or single pellet retrieval tasks; again this functional recovery was associated with CST plasticity in the pellet retrieval rehabilitation group (Starkey et al., 2011). In contrast to the previously described study however, task-specific rehabilitation resulted in functional improvements in all behavioural tasks assessed rather than task-specific improvements (Starkey et al., 2011). These contrasting findings may well be due to the different injury models used in the two studies; with a number of spinal pathways lesioned in the dorsolateral quadrant transection of the first study, whilst a discrete unilateral lesion of only the CST was employed in the second. Task-specific rehabilitation in both studies involved tasks heavily reliant on CST function and, therefore, was likely to enhance and guide plasticity of the CST in particular. In the scenario of only the CST being injured this was always likely to lead to only beneficial effects, whereas when other pathways are injured the spontaneous plasticity and recovery of these additional pathways may have been detrimentally affected by the targeted rehabilitation of the CST. In recent years, neurorehabilitation strategies have been developed involving electrical and/or chemical activation of spared spinal circuitry to mimic, or even complement, the effects of behavioural rehabilitation. This can involve techniques such as repeated epidural electrical stimulation or administration of exogenous neurotransmitters or neurotransmitter receptor agonists, such as serotonin or dopamine. Using such strategies it has been shown that even after complete transection of the spinal cord the combination of electrical, chemical and exercise induced activation of intact spinal circuitry below the lesion enables the taking of fully weight

supported steps on a treadmill (Courtine et al., 2009). Furthermore, this strategy can promote the formation of *de novo* spinal relay circuitry through spared spinal tissue, resulting in significant functional improvements (van den Brand et al., 2012).

The use of neurorehabilitative techniques alone can have potent effects on plasticity and spinal functionality, but are likely to be even more effective if carefully combined with factors targeted at enhancing the endogenous response to neurorehabilitation. As mentioned above, the combination of epidural stimulation with neurotransmitter receptor agonists and physical training has resulted in dramatic effects on plasticity and function. A number of other studies have shown additive or synergistic effects of some therapeutic interventions when combined with neurorehabilitation, whilst some interventions show no additional benefits from this combination. For example, Nogo-A neutralising antibodies and treadmill locomotor training show therapeutic benefits independent of one another. When combined however, they resulted in highly variable, poorly coordinated gait in spinal injured rats (Maier et al., 2009). However, the combination of task-specific behavioural rehabilitation and the CSPG-degrading enzyme, chondroitinase ABC (ChABC), resulted in additive functional benefits (Garcia-Alias et al., 2009b). Interestingly, ChABC combined with non-specific behavioural rehabilitation (i.e. increased activity, but not in a specific task) was detrimental to skilled reaching, but ladder walking and grip-strength were improved (Garcia-Alias et al., 2009b), again highlighting that rehabilitative strategies can lead to both positive and negative effects depending on how they are targeted. Findings such as these highlight the importance of combining strategies with effects that are likely to complement one another. Individual components should therefore be tailored around one another in order to optimise the additive or synergistic effect the full combination will have. Additionally, the timing of each component is likely to have an effect on the

success of a combination strategy. For example, the combination of ChABC and rehabilitation appears to be most effective if rehabilitation is initiated at the same time as, or soon after, treatment with ChABC. It is suggested that treatment with ChABC creates a more plastic environment in the spinal cord and, therefore, the effects of rehabilitation are amplified by this enhanced plastic state and can guide and refine the enhanced plasticity towards the restoration of useful function. Encouragingly, this combination was shown to remain effective even if treatment was delayed until 4 weeks post-injury (Wang et al., 2011a). A recent study has further demonstrated the importance of timing in the administration of a combination therapy, highlighting that the combination of anti-Nogo-A, ChABC and behavioural rehabilitation is most effective if anti-Nogo-A is administered acutely followed by ChABC treatment and rehabilitation once anti-Nogo-A treatment has ceased (Zhao et al., 2013). Mechanistic and neuroanatomical insights into the actions of individual and combined neurorehabilitative therapeutic strategies may be of key importance in the design of future experimental and clinical treatment protocols.

1.5.7 Disruption of inhibitory chondroitin sulphate proteoglycans

The potent inhibitory effects of CSPGs on axonal growth, as well as their dramatic and sustained upregulation after injury, identify these molecules as an obvious target for therapeutic intervention. Accordingly, there have been a vast number of studies involving the disruption of CSPGs, using a variety of techniques. Whilst enzymatic degradation using the bacterial enzyme ChABC has been by far the most widely used of these techniques (discussed in detail below), a number of other approaches to CSPG disruption have also been employed.

1.5.7.1 Alternative strategies for CSPG disruption

Decorin, a member of the small leucine-rich proteoglycan family, has been shown to reduce reactive gliosis and the production of CSPGs following injury to the brain (Logan et al., 1999a) or spinal cord (Davies et al., 2004), resulting in enhanced axonal growth through the injury site (Davies et al., 2004). It is thought that these effects are due to the inhibitory effects of decorin on TGF- β and epidermal growth factor (EGF) signalling (Yamaguchi et al., 1990; Santra et al., 2002; Davies et al., 2004), both of which promote astrocyte activation and proliferation, resulting in CSPG deposition (Rabchevsky et al., 1998; Asher et al., 2000). *In vitro* studies have also shown that decorin can have a direct effect on neurons, boosting their ability to extend axons in an inhibitory environment (Minor et al., 2008). A DNA enzyme targeted at inhibiting xylosyltransferase-1 (XT-1), the enzyme responsible for glycosylation of CSPGs, reduces CS-GAG chains *in vitro* and *in vivo* and, like decorin, reduces CSPG levels at the glial scar (Grimpe and Silver, 2004; Grimpé et al., 2005; Hurtado et al., 2008). The inhibition of XT-1 in models of SCI has been shown to increase axonal growth around and through the glial scar (Grimpe and Silver, 2004; Hurtado et al., 2008). CSPG synthesis can also be modified by targeting enzymes responsible for sulphation of CS-GAGs and genetic modification has allowed the knockdown (Nadanaka et al., 2011) or knockout (Ohtake-Niimi et al., 2010) of some of these enzymes in mice. The sulphation motifs of CS-GAGs are thought to act as recognition elements for many CSPG interactions (Gama et al., 2006; Shen et al., 2009; Coles et al., 2011) and thereby confer many of their inhibitory actions. It would therefore, be interesting to investigate how axonal regeneration is affected following SCI in the genetically modified mice mentioned above; particularly as antibody blockade of a specific sulphation motif, CS-E, has been shown to significantly enhance axonal regeneration following optic nerve

injury (Brown et al., 2012). Finally, genetic knock-out of the link protein Crtl 1 results in attenuated formation of CSPG-rich perineuronal nets (PNNs) during development. This allows enhanced axonal sprouting into nuclei containing cell bodies that would normally be surrounded by inhibitory PNNs, as indicated by the sprouting of sensory fibres into the partially denervated cuneate nucleus (Carulli et al., 2010). Therefore, strategies which disrupt CSPG-rich PNNs may also potentially enhance plasticity following SCI.

1.5.7.2 ChABC neutralises the inhibitory effects of CSPGs on axon growth

Whilst a number of promising strategies for modifying CSPGs have been developed in recent years, ChABC has been the most successful, and therefore the most widely used, of these strategies to date. ChABC cleaves the CS-GAG chains from the CSPG core protein (Yamagata et al., 1968), thereby preventing the majority of the key interactions through which CSPGs mediate their inhibitory effects (mechanisms underlying the effects of ChABC are discussed in detail in section 5.2). The first observations that ChABC-mediated degradation of CSPGs lead to growth permissive effects in CNS neurons were made *in vitro*. For example, ChABC treatment of adult glial scar explants, known to be a strongly inhibitory substrate expressing high levels of CSPGs (McKeon et al., 1991), resulted in a dramatic increase in retinal ganglion cell (RGC) axonal growth over the explants (McKeon et al., 1995). ChABC treatment also leads to significantly increased axonal outgrowth of DRG neurons in the presence of ECM derived from growth-inhibitory astrocytic cell lines (Smith-Thomas et al., 1994) or oligodendrocyte lineage cells (Asher et al., 2002). Additionally, treatment with ChABC was shown to significantly enhance DRG axonal outgrowth on normal and injured spinal cord cryosections (Zuo et al., 1998b). Following these promising *in vitro* results, the effects of ChABC *in vivo* were assessed. Lemons *et al.* (1999) demonstrated that

exogenous application of ChABC at the site of a spinal contusion injury resulted in cleavage of the scar-associated CSPGs. It was subsequently shown that ChABC treatment of a thoracic hemisection injury resulted in the regeneration of axons from Clarke's column into a peripheral nerve graft, whilst neither vehicle nor BDNF treatment could encourage the same axonal population to grow (Yick et al., 2000). Further to this, significant CNS axonal regeneration was also achieved following infusion of ChABC to treat axotomy of the nigrostriatal tract in the rat brain, resulting in the regeneration of dopaminergic axons across the lesion and back to their striatal targets (Moon et al., 2001).

1.5.7.3 ChABC promotes functional recovery

Key findings showed that not only could ChABC promote regeneration, but that it could significantly enhance functional recovery (Bradbury et al., 2002). In this study adult rats received a dorsal column crush at cervical level and this was followed by intrathecal infusion of ChABC for 10 days. In addition to regeneration of sensory and CST axons, this resulted in significant functional improvements in a number of behavioural assessments, including grid walking, sensing and removal of sticky tape, beam walking and gait analysis. Electrophysiological recordings in this study also showed that intrathecal ChABC infusions significantly enhanced CST functionality. Whilst this was the first study to show the effects of ChABC on functional recovery in an *in vivo* model of SCI, these effects have now been replicated and further assessed in a variety of injury models. ChABC treatment has enhanced recovery of locomotion, proprioception and skilled forelimb reaching following cervical dorsal column injury (Bradbury et al., 2002; Garcia-Alias et al., 2008), as well restoring forelimb symmetry (Yick et al., 2004) and hemidiaphragm function following a unilateral hemisection (Alilain et al., 2011). Repeated intrathecal delivery of ChABC also resulted in improved locomotor and

bladder function following a severe compression injury, which represents a more clinically relevant model of SCI (Caggiano et al., 2005). Importantly, ChABC has now also been shown to promote functional recovery in larger animals, where intrathecal delivery of ChABC enhanced recovery of skilled locomotion and kinematic measures of hindlimb function following thoracic hemisection injury in adult cats (Tester and Howland, 2008).

ChABC has also become increasingly used as part of a combination to enhance spinal cord repair in experimental SCI studies. For example, the combination of repeated ChABC infusions with a Schwann cell seeded guidance channel and OEC transplants to treat a complete thoracic transection injury in rats has been shown to enhance locomotor and sensory function (Fouad et al., 2005). This functional recovery was associated with the regeneration of myelinated fibres through the Schwann cell guidance channel and back into the host tissue and all outcome measures were notably improved by the inclusion of ChABC in the combination treatment compared with just Schwann cell and OEC combination treatment. This triple combination has also been shown to improve the recovery of bladder function in the same injury model (Fouad et al., 2009). In another combinatorial treatment study the addition of ChABC to a combination of a peripheral nerve graft and GDNF was shown to significantly enhance the number of regenerating fibres able to grow beyond the graft and reintegrate into the host tissue, resulting in improved locomotor function in the forepaw affected by the C5 hemicontusion injury model employed (Tom et al., 2009b). ChABC combined with just growth factor has also been shown to be a successful combination treatment (Massey et al., 2008). In this study both ChABC and NT-3 treatments alone promoted modest levels of axonal sprouting from microtransplanted DRG cells into a denervated dorsal column nucleus (DCN) in which CSPGs had been shown to be upregulated following

disruption of the fasciculus gracilis at cervical or thoracic level. Combining both ChABC microinjection and lentiviral vector induced NT-3 expression in the DCN dramatically increased axonal growth into the denervated DCN due to disruption of the inhibitory CSPGs (ChABC) and the provision of a growth guidance cue (NT-3). Many further studies involving the use of ChABC as part of a combination treatment have been carried out, such as those examining the combination of ChABC with rehabilitation and/or anti-Nogo-A treatment which have already been discussed (Garcia-Alias et al., 2009b; Wang et al., 2011a; Zhao et al., 2013). Studies involving combination of ChABC with a peripheral nerve graft (Alilain et al., 2011) or NPCs and growth factors (Karimi-Abdolrezaee et al., 2010) will be discussed in the section below.

1.5.7.4 ChABC promotes plasticity

Initially the effects of ChABC on functional recovery were thought to be largely due to axonal regeneration. More recently however, its effects on plasticity have become the focus of much attention since CSPGs are known to be important in restricting neuronal plasticity (Pizzorusso et al., 2002; Fox and Caterson, 2002; Berardi et al., 2004; Rhodes and Fawcett, 2004; Bartus et al., 2012). In particular, CSPGs surrounding neuronal cell bodies in the form of PNNs have been shown to have important roles in maintaining synaptic stability and restricting plasticity throughout the adult CNS (Celio and Blumcke, 1994; Pizzorusso et al., 2002; Murakami and Ohtsuka, 2003; McRae et al., 2007). For example, following ChABC-mediated degradation of PNNs in the visual cortex of adult rats, mono-ocular deprivation induced shifts in ocular dominance at a developmental time point when organisation of the visual cortex is normally fixed (Pizzorusso et al., 2002). Indeed, the deposition of CSPG-containing PNNs has been shown to closely correlate with the closure of the “critical period” of development in a number of brain regions, during which plasticity induced changes shape the final

neuronal organisation (Pizzorusso et al., 2002; Berardi et al., 2003; McRae et al., 2007). In light of this it is not altogether surprising that the upregulation of CSPGs following SCI further limits plasticity, and that ChABC treatment can therefore significantly enhance plasticity. In one of the first studies to show this, aberrant sprouting of the abolished CST was seen rostral to a dorsal column crush injury, whilst there was also robust sprouting of the intact raphe-spinal system caudal to the injury site in ChABC-treated animals (Barritt et al., 2006). Furthermore, another study showed that degradation of PNNs in the cuneate nucleus following cervical dorsal column transection led to sprouting of spared sensory fibres into the denervated areas of the nucleus (Massey et al., 2006). Additionally, a single intra-spinal injection of ChABC has been shown to promote robust sprouting of intact serotonergic fibres following either a hemisection or contusion injury (Tom et al., 2009a). The role of ChABC in enhancing plasticity was highlighted in a study using a combination of a NPCs, growth factors and ChABC to treat a spinal compression injury. This combination treatment induced robust, aberrant sprouting of intact CST and serotonergic fibres and the key role of ChABC in this plasticity was indicated by the distinct lack of this sprouting in animals which did not receive ChABC as part of their combination therapy (Karimi-Abdolrezaee et al., 2010)

Importantly, plastic changes induced by ChABC treatment are also linked with functional improvements. ChABC-induced plasticity has been shown to result in full recovery of forelimb sensory function following a spared root injury involving rhizotomy of multiple dorsal roots conveying sensory information from the forelimb (C5, C6, C8, T1) and leaving the C7 root intact. Electrophysiological assessments demonstrated novel functional connectivity between spared C7 fibres and second order neurons in deafferented spinal segments following ChABC treatment. Enhanced

plasticity of fibres in the spared C7 root was thought to mediate this recovery and this was further supported by evidence of anatomical sprouting from C7 afferents and a lack of regeneration from any of the injured roots (Cafferty et al., 2008). In a recent study the combination of ChABC and a peripheral nerve graft bridging a C2 lateral hemisection resulted in long-range regeneration, plasticity of spared axonal tracts and plastic remodelling of spinal circuitry. Again ChABC-mediated plasticity was shown to play an important functional role as even ChABC treatment alone induced sprouting of spared serotonergic fibres, resulting in a partial restoration of function in the affected hemidiaphragm (Alilain et al., 2011). Functional recovery in the denervated forelimb has also been associated with robust sprouting of spared CST fibres following unilateral pyramidotomy and ChABC treatment (Starkey et al., 2012). Delivery of ChABC to the spinal cord has also been shown to result in reflex reorganisation and enhanced functional recovery following incorrect re-apposition of transected peripheral nerves (Galtrey et al., 2007; Bosch et al., 2012).

Whilst plasticity and regeneration induced by ChABC degradation of CSPGs are likely to be key contributing factors to the functional recovery observed following ChABC treatment in many models of SCI, it is also interesting to note that CSPGs have been shown to be capable of blocking conduction in intact axons (Hunanyan et al., 2010). Application of ChABC can alleviate this conduction block and this may well contribute to the improved function observed in many studies. ChABC has also been shown to prevent atrophy of corticospinal neuronal cell bodies following either acute intracerebroventricular or local intrathecal administration to treat a thoracic dorsal column crush injury (Carter et al., 2008), and has even been shown to reverse chronic atrophy of rubrospinal neuronal cell bodies following cervical axotomy (Carter et al., 2011). These results suggest that some of the functional improvements resulting from

ChABC treatment may be partially mediated by neuroprotective mechanisms. These multi-faceted effects of ChABC combine to promote robust functional repair of the injured spinal cord, highlighting the disruption of CSPGs via ChABC-mediated degradation as a particularly promising therapeutic intervention for the treatment of SCI. This strategy will therefore be the focus of much of this thesis.

1.6 Aims of the thesis

The primary aims of this thesis are: i) to characterise both pathological and functional changes that occur in a number of clinically relevant models of spinal cord injury in rats, with a particular focus on using electrophysiological techniques to assess changes in the functionality of individual spinal pathways and ii) To assess the therapeutic potential of chondroitinase ABC in each of these clinically relevant injury models, delivered using a novel gene therapy approach.

The findings of these studies are presented in the following chapters of experimental data:

Chapter two characterises the pathological and functional changes that occur following a thoracic contusion injury of moderate severity. A variety of techniques are employed to examine the temporal pattern of these changes, but focus is particularly directed at conduction properties of long-distance, sensory dorsal column axons as well as the processes of demyelination and remyelination in these axons.

Chapter three assesses the therapeutic potential of using a lentiviral vector to deliver ChABC to a moderate thoracic contusion injury. Firstly, comparisons are made between enzyme delivery and delivery using three different lentiviral vectors in order to select

the optimal vector that results in the most widespread and long-term digestion of CSPGs. The lentiviral vector with the most desirable treatment profile is then used to treat a moderate thoracic contusion injury and its therapeutic efficacy is assessed using a number of anatomical, behavioural and electrophysiological techniques established in the previous chapter.

Chapter four focuses primarily on characterising the functional changes that take place following either a moderate cervical contusion injury or a severe thoracic contusion injury, and whether ChABC gene therapy can significantly enhance functional recovery in these clinically relevant injury models. A variety of electrophysiological techniques are employed in order to assess functionality of both motor and sensory pathways, whilst the use of a cervical contusion injury model allows for the implementation of a wide variety of behavioural techniques in order to assess behavioural changes in function.

Thus, this thesis focuses on the development of a novel gene therapy approach for the administration of chondroitinase ABC and assesses the efficacy of this promising therapeutic in a number of clinically relevant models of spinal cord injury. Key findings, as well as the possible mechanisms of action and potential clinical applications of this treatment, will be discussed in chapter five.

Chapter Two

2 CHAPTER 2:

Characterising the temporal pattern of functional and anatomical changes in a clinically relevant contusion model

This chapter is an expanded version of a published paper: James *et al.* (2011). Conduction failure following spinal cord injury: functional and anatomical changes from acute to chronic stages. *J Neurosci* **31**(50):18543-18555.

2.1 Introduction

SCI can lead to severe and permanent deficits in motor, sensory, and autonomic function. There is now a wealth of experimental studies showing improved function following various therapeutic interventions, such as targeting inhibitory myelin (Cafferty *et al.*, 2010; Zorner and Schwab, 2010) and CSPGs (Bradbury *et al.*, 2002; Alilain *et al.*, 2011; Bradbury and Carter, 2011) or by growth promoting strategies (Alto *et al.*, 2009) and targeting microtubule dynamics (Hellal *et al.*, 2011). As many promising treatments are now progressing towards clinical trials, it is important for experimental studies to focus on clinically relevant injury models. The initial insult to the spinal cord can normally be classified as a contusion, compression or laceration injury (Bunge *et al.*, 1997), with the majority of human injuries involving contusion of the spinal cord (Norenberg *et al.*, 2004). Thus, experimental animal models of spinal contusion provide a clinically relevant system with which to study SCI.

Experimental contusion devices have evolved from early simplistic weight-drop devices, first used in dogs (Allen, 1911) and then rat (Gale *et al.*, 1985; Noble and

Wrathall, 1985;Wrathall et al., 1985), to more precisely controlled devices which give graded and reproducible injuries and incorporate the use of mechanical sensors to monitor parameters of the impact (Bresnahan et al., 1987;Gruner, 1992;Stokes et al., 1992;Stokes, 1992;Young, 2002) and to deliver force-defined injuries (Scheff et al., 2003). The three most widely used such devices are the New York University (NYU) weight drop device, the Ohio State University (OSU) electromagnetic spinal cord injury device (ESCID) and the Infinite Horizon (IH) impactor. The NYU device is a more sophisticated version of previous weight drop contusion devices, involving the controlled drop of a specific weight (10g is the standard weight, but this can be customised) from a user specified height (6.25, 12.5, 25, 50 or 70 mm) in order to vary the severity of injury (Gruner, 1992). The particular advantage of this device over other weight drop devices was the inclusion of sensors that could measure important injury parameters including the actual height of drop, velocity at impact and cord compression. However, as the impact was controlled solely by gravity the impact weight bounces on the spinal cord following initial impact causing a second impact when it falls again (Gruner, 1992). Additionally, whilst there is direct correlation, with little variation, between height of drop and velocity of impact using this device, there is a relatively high degree of variability in the correlation between height of drop and spinal cord compression, suggesting there will be variability in the severity of impairment. Indeed, in this original study this resulted in one animal group that received injuries from a specific weight drop height receiving notably more severe injuries than an additional group that was injured using the same injury parameters (Gruner, 1992). The OSU device avoided these issues by having an impactor probe controlled by an electromagnetic driver which drives the impactor probe into the spinal cord until it reaches a user-specified displacement value before almost immediately (4-5 ms delay)

withdrawing it to a position well above the spinal cord (Stokes et al., 1992; Stokes, 1992). In addition to preventing the issue of multiple impacts associated with weight drop devices, this device also provides detailed information on a number of injury parameters including actual displacement achieved, velocity of impact and the force of impact. However, there are also some small disadvantages associated with this device. Firstly, in order to specify the injury severity by cord displacement the impactor probe must be in contact with and applying a small degree of pressure to the exposed spinal cord (Stokes, 1992), raising the possibility of a small pre-injury which could lead to variability in the subsequent experimental injury. Additionally, devices which rely on displacement to control injury severity are susceptible to variability caused by small movements of the specimen during the impact event (such as those associated with breathing and blood flow) (Scheff et al., 2003). The more recently developed IH impactor allows the user to define the injury severity by selecting the force of impact, thereby preventing any variability associated with small movements of the specimen as well as avoiding the necessity for any contact with the spinal cord prior to injury (Scheff et al., 2003). As with each the previously described devices, the IH impactor provides important information on injury parameters such as actual force, displacement, and velocity. The advancement of sophisticated contusion devices has been invaluable in gaining detailed information about the histopathology of contusion lesions. Rodent contusion models, particularly the rat, bear a striking similarity to human SCI pathology (Metz et al., 2000a). For example, both rats and humans develop fluid-filled cavities surrounded by a rim of spared white matter subsequent to a spinal contusion (Figure 2.1) (Nurick et al., 1970; Bunge et al., 1993; Basso et al., 1996; Quencer and Bunge, 1996; Norenberg et al., 2004; Hagg and Oudega, 2006) with the presence of cystic cavitations divided by tissue bridges containing axons (Hill et al., 2001; Radojicic et al.,

2005), similar to the septated syringomyelia observed in a human injury (Padilla, 1982;Lederhaus et al., 1988). Pathological processes such as haemorrhage, Wallerian degeneration, axonal die-back, demyelination, Schwannosis, and glial scarring also occur in both human SCI and rat contusion models (Noble and Wrathall, 1989;Guest et al., 2005;Totoiu and Keirstead, 2005;Hagg and Oudega, 2006).

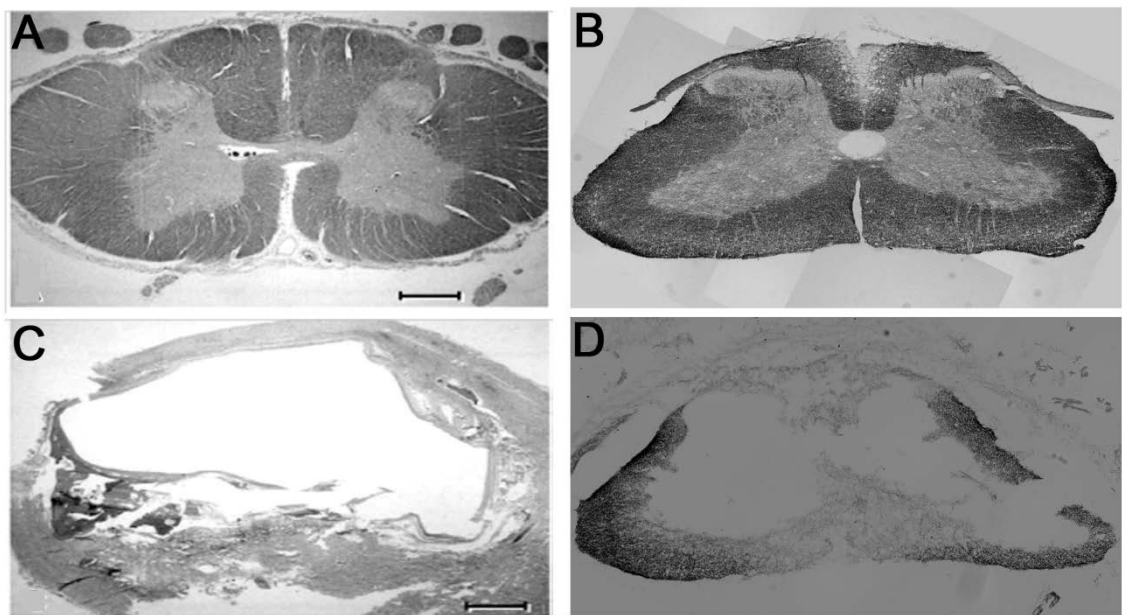


Figure 2.1. Comparison of a human and a rat spinal contusion injury. Representative images of transverse sections of uninjured human (A) and rat (B) cervical spinal cord. (C+D) Following spinal contusion injury large, fluid-filled cavities form in the spinal parenchyma of both species, leaving a spared rim of white matter and dense scarring surrounding the cavity border. Images of human spinal cord taken and adapted from Norenberg et al., 2004.

While many of the pathological processes that occur following SCI have been well documented, a key goal in SCI research is to understand mechanisms that underlie changes in function following SCI. Behavioural testing has been the most commonly employed technique for assessing functional changes (Basso, 2004), with a wide variety of existing behavioural techniques commonly used to assess different aspects of function following SCI, such as locomotion (Basso et al., 1995;Basso et al., 2006), grip strength (Anderson et al., 2004), skilled reaching (Whishaw et al., 1998), proprioception (Fehlings and Tator, 1995) and sensory function (Detloff et al., 2010). Behavioural assessment of function however, reveals little about the mechanisms underlying any observed functional changes (Bradbury and McMahon, 2006). Electrophysiological assessment of function has the advantage that it can reveal specific information about the integrity and conductivity of spinal fibres within specific spinal pathways. A number of previous studies have used non-invasive, magnetic stimulation paradigms for evaluating axon conduction following experimental contusion injury, such as transcranial magnetic motor evoked potentials (tcMMEPs)(Metz et al., 2000a;Cao et al., 2005b), magnetically evoked interenlargement responses (MIERs)(Beaumont et al., 2006) and somatosensory evoked potentials (SSEPs) (Metz et al., 2000a;Ellingson et al., 2008) for the evaluation of supraspinal, propriospinal and sensory spinal axon conduction, respectively. Whilst minimally invasive electrophysiological techniques allow repeated assessment of the same animal, meaning that functional changes can be measured in the same animal over a post-injury time course, the indirect nature of these techniques means that they are naturally associated with relatively high levels of variability. The use of more invasive electrophysiological techniques reduces this variability, but the invasive nature often means that experiments must be terminal and therefore functional changes cannot be monitored in the same animal over a time

course. A relatively small number of studies have used direct, invasive electrophysiological techniques to assess the effects of a contusion injury. For example, Hains and colleagues (2004) looked at compound action potentials (CAPs) of dorsal column axons 4 weeks after SCI via direct, extracellular stimulation of the dorsal columns caudal to a contusion injury whilst recording CAPs rostral to the lesion site. In this study the changes in CAP characteristics between untreated, treated, and control individuals correlated well with the results of behavioural assessment (Hains et al., 2004). Lee and colleagues (2007) assessed SSEPs and MEPs, but unlike the minimally invasive studies mentioned above, they stimulated and recorded directly from the sensorimotor cortex whilst either recording from the grey matter of the L1 spinal cord (MEPs) or stimulating the sciatic nerve (SSEPs). 9 weeks post-SCI the amplitude of both SSEPs and MEPs in untreated individuals was reduced to approximately 50% of control values and latencies were greatly increased (Lee et al., 2007). Further invasive electrophysiological assessments of experimental contusion injuries have involved the recording of cord dorsum potentials, H-reflexes and monosynaptic reflexes (Thompson et al., 1992), sensory- and motor-evoked potentials (Cerri et al., 2012) and the mapping of thalamic receptive fields following visceral or cutaneous stimulation (Hubscher and Johnson, 2006). Recordings from these studies could then be used for evaluation of hindlimb reflexes, temporal changes in conductivity of long distance sensory and motor spinal projections, and supraspinal pain processing, respectively. Invasive paradigms can reveal detailed and specific functional information such as integrity and conduction velocities of individual fibres (Boucher et al., 2000) and function of specific spinal pathways (McMahon and Wall, 1983;Hubscher and Johnson, 2006) or demonstrate functional plasticity (Massey et al., 2006;Cafferty et al., 2008). There is a paucity of information however, regarding how individual fibres in the spinal cord are affected by

contusion injury in terms of their conduction properties. This is of particular clinical importance since, despite the classic pathology and well-established histological observations of residual spared axons, very little is known about the *function* of surviving axons in spinal contusion injuries. For example, whether axons in the spared rim of white matter are viable and capable of conducting and whether there are changes in functionality over time. It is not known whether these axons stabilise within the acute post-injury phase or whether changes continue into chronic stages and, crucially, whether function can be restored to these axons in a chronic injury.

The aim of this chapter is to perform detailed functional and anatomical assessments in order to address the issues outlined above. A novel electrophysiological preparation, which allows comparisons of the functionality of individual sensory fibres above and below the site of a spinal cord lesion, will be used to obtain detailed information about the conduction properties of long distance ascending dorsal column axons. The temporal pattern of conduction failure and changes in conduction properties following spinal contusion injury in adult rats over an acute to chronic time course (from 1 day to 6 months) will be assessed using this technique. Additionally, changes in conduction will be compared and correlated with quantitative electron microscopy plus further histological and behavioural analyses in order to elucidate potential mechanisms for the observed post-injury functional changes. The combination of these techniques will be used to demonstrate changes in the functional properties of a major spinal pathway following SCI. Results showed a complete conduction block of ascending dorsal column axons in the acute post-injury phase, followed by a period of improved conduction in the sub-acute phase which plateaus and then remains stable into the chronic stages of injury. Additionally, cooling the lesion site enhanced conduction across the contusion injury, thus revealing a population of axons that are viable but

unable to conduct under normal physiological conditions. This phenomenon was still apparent in the chronic stages of SCI. The findings of this chapter document the time course over which viable, but initially non-conducting axons, regain useful functionality and reveal a population of surviving axons that remain chronically unable to conduct under normal physiological conditions, identifying this axonal population as a prime target for therapeutic strategies to restore function following SCI.

2.2 Materials and methods

2.2.1 Animals

Adult female Sprague-Dawley rats ($n = 70$; 200-220g; Harlan laboratories) were used (housed under a 12 hour light/dark cycle with free access to food and water). At various post-injury time points animals were removed for electrophysiological, histological or morphological studies ($n = 5/6$ per time point for electrophysiology; $n = 4$ per time point for histology; $n = 4$ per time point for electron microscopy [EM]; Fig. 2.2). Behavioural testing was carried out throughout the study such that all animals ($n = 70$) were included in the first time point with fewer animals at each subsequent time point as they were assigned to the other outcome measures, so that by the final testing weeks (12 weeks and 6 months) there were $n = 23$ and $n = 9$, respectively. All experimental procedures were performed in accordance with the U.K. Animals (Surgical Procedures) Act 1996.

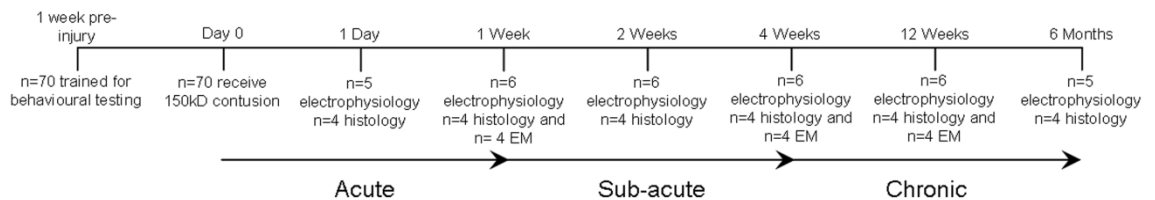


Figure 2.2. Timeline outlining experimental design. At a number of post-injury time points, spanning acute to chronic stages of spinal cord injury, animals were removed for terminal electrophysiological experiments, for histological assessments or for electron microscopy (EM). Behavioural testing was carried out on all remaining animals throughout the study. This allowed a detailed assessment of functional changes as well as pathological and morphological processes that occur over time following contusion injury.

2.2.2 Contusion injury surgery and post-operative care

Animals were anaesthetised using a mixture of ketamine (60mg/kg) and medetomidine (0.25mg/kg; administered i.p.), their backs were shaved and cleansed with iodine and core temperature was maintained close to 37°C using a self-regulating heated blanket. Single doses of 0.05mg/kg buprenorphine and 5mg/kg carprofen were given subcutaneously at the time of induction and the morning after surgery. Laminectomies were performed at vertebral level T10, the vertebral column was stabilised using Adson forceps and the impactor probe was positioned 2-4mm above the spinal cord. An impact force of 150 kilodyne (kdyne) was delivered to the exposed spinal cord through the intact dura with an Infinite Horizons impactor (Precision Systems Instrumentation, Lexington, KY) to create a moderate severity contusion injury. This severity was chosen since pilot studies (data not shown) revealed that more severe (200 kdyne) injuries did not spare enough dorsal column white matter to perform electrophysiological assessments in the ascending dorsal column projection and more mild (100 kdyne) injuries spared too much of the dorsal columns, making it difficult to observe any degree of change in conduction over time. This severity also mimics the >50% of human injuries that are “incomplete” i.e. where some white matter tissue is spared which contains uninjured axonal projections, thus making this model very relevant to the clinical situation. Overlying muscle and skin were sutured, anaesthesia was reversed using atipamezole hydrochloride (1mg/kg administered i.p.) and animals recovered in cages placed on a heated blanket overnight. Saline (3-5ml) and enrofloxacin (5mg/kg) were given subcutaneously daily for 3 and 7 days, respectively, post-injury. Bladders were manually expressed twice daily until reflexive emptying returned (typically 6-9 days post-injury).

2.2.3 Behavioural assessment

2.2.3.1 BBB locomotor scoring

Open field hindlimb locomotor function was assessed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Briefly, this involved placing the animal in a circular open field (diameter of 1 metre) and assessing both hindlimbs during locomotion (over a 4 minute session) for individual joint movements, paw placement, weight support, consistency of stepping and hindlimb/forelimb coordination, level of toe clearance during stepping, and overall trunk stability. Scores were calculated according to the 22 point (0-21) BBB scale for each hindlimb and averaged to give each animal an overall score. Testing was carried out on days 1, 3, 5 and 7 post-injury and weekly thereafter for 12 weeks, with a final assessment at 6 months.

2.2.3.2 Horizontal Ladder

Animals were trained daily for 1 week prior to injury to run across a 1 metre long horizontal ladder with unevenly spaced rungs. On the final day of training animals were recorded using a Sony DCR-SX30E Handycam. Recordings were later analysed in slow motion, allowing quantification of the total number of hindlimb footslips and misses during the course of three runs across the ladder, giving each animal a baseline score. Any form of miss or footslip which resulted in the entire paw going below the level of the rung was counted as a score of 1. This testing procedure and analysis was then repeated once a week post-injury for 12 weeks, beginning at day 7, with a final assessment at 6 months.

2.2.4 Electrophysiological studies

2.2.4.1 *Measuring percentage conduction through the lesion and conduction velocity*

Electrophysiological assessments were performed on the ascending dorsal column projection, a major spinal pathway that projects the length of the dorsal columns in the gracile fasciculus, conveying sensory information about hindlimb and hindpaw placement. Terminal electrophysiological assessments were performed at 1d, 1wk, 2wk, 4wk, 12wk, and 6mth post-injury time points (n=5/6 per time point), to assess changes in conduction across a contusion injury over acute to chronic stages of injury. To calculate the appropriate number of animals for each group an *a priori* power analysis was carried out (G*Power v 3.1.7) using an estimated effect size of 1.3 (based on previous observations), revealing that a total sample size of 24 (n=4 per time point) should be sufficient to detect statistical differences ($\alpha=0.05$, $1-\beta=0.95$, $d=1.3$). For the duration of each electrophysiological experiment animals were deeply anaesthetised with urethane (1.25g/kg; administered i.p.) and depth of anaesthesia was regularly assessed by monitoring pedal withdrawal reflexes and respiratory rate. Core temperature was maintained close to 37°C using a self-regulating heated blanket. A laminectomy was performed to remove the dorsal portions of vertebrae T7-L5. The dura mater was removed from the spinal cord, exposed nervous tissue was covered with mineral oil and silver-wire stimulating electrodes were placed over the midline approximately 5mm rostral and caudal to the lesion site. Tiny filaments were teased from dorsal roots L3-S1 (both left and right hand sides) and individually mounted on silver-wire recording electrodes, allowing for the recording and quantification of single units (activity of single nerve fibres) from each of these filaments whilst stimulating either above or below the lesion site (Fig. 2.3). Firstly, the number of antidromically activated single units present in each filament was counted whilst stimulating caudal to the lesion (5-10

single units were normally present per filament); this was then repeated whilst stimulating rostral to the lesion to calculate the percentage of nerve fibres capable of conducting through the lesion. In all electrophysiological experiments the dorsal columns were stimulated using 0.2 ms duration square wave pulses at a frequency of 1 Hz and an incrementally increasing intensity (0-800 μ A, activation tended to be maximal by 400-500 μ A). During recording sessions stimulation was continuous and lasted until all units in the filament being recorded from had been identified. At the end of each experiment measurements were made of inter-electrode distances to allow for the calculation of conduction velocities. Conduction velocities were calculated for each single unit recorded and then averaged for each animal (mean \pm SEM values were then calculated for each time point).

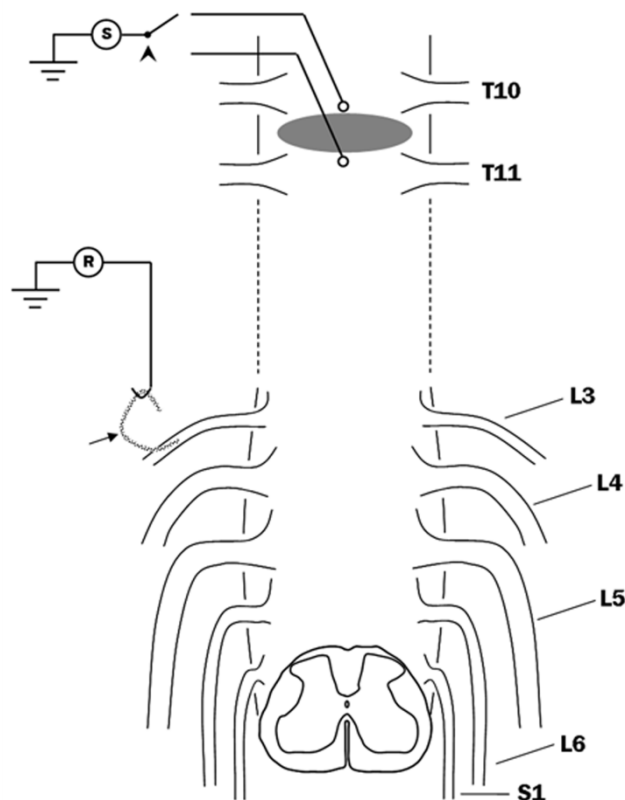


Figure 2.3. Schematic diagram illustrating the protocol used for assessing conduction failure. Antidromically activated unitary activity was recorded from teased dorsal root filaments

(arrow) on both sides of the spinal cord from dorsal roots L3-S1 whilst first stimulating caudal to the injury site (grey oval) and then rostral to the injury site (using switch, arrowhead); allowing a quantitative measure of the percentage of fibres capable of conducting across a contusion injury. S = stimulating electrode, R = recording electrode.

2.2.4.2 Cooling of the lesion

In a model of primary demyelination in the rat it has previously been shown that cooling of the demyelinated region restored conduction in some previously non-conducting fibres (Bostock et al., 1978; Smith et al., 2000). In order to test the hypothesis that following SCI there may be some fibres that are spared but unable to conduct across the injury we cooled the lesion site using cold mineral oil and noted the addition of any extra single units that were recruited due to this cooling whilst stimulating above the lesion. Cold mineral oil (in 20ml plastic syringes that had been stored at -4°C) was carefully applied around the lesion site to progressively cool the injury. Temperature was closely monitored using a temperature probe placed adjacent to the lesion site and connected to a tele-thermometer unit (Yellow Springs Instrument Co., Inc.). Temperature was gradually decreased by applying small volumes of cold mineral oil at a time and a note was made of the temperature at which any additional units were added. This preparation was continued until the temperature had cooled from 37°C to 15°C. The final 3-4 filaments that had been recorded from in each animal were tested for any effect of cooling in all electrophysiological experiments.

2.2.5 Behavioural and electrophysiological correlations

Correlations of behavioural and electrophysiological data were performed on individual animals in the study (n = 4 animals per time point that underwent electrophysiological assessments; i.e. n = 24 for BBB tested animals and n = 20 for ladder tested animals since they did not perform the ladder test at the 1 day post-injury time point).

Percentage conduction values of individual animals were plotted against their behavioural scores (for BBB values and footslip errors) and correlation coefficients were calculated using Pearson's correlation test.

2.2.6 Histology

2.2.6.1 Tissue preparation

Animals were deeply anaesthetised with sodium pentobarbital (Euthatal; 80 mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Immediately after perfusion a section of spinal cord was removed (~10mm) with the lesion epicentre located centrally; this was then post-fixed in 4% PFA in 0.1 M PB for 2 hours at 4°C before being cryoprotected in 20% sucrose in 0.1 M PB for 48 hours. The tissue was then embedded in OCT and frozen before being cut into serial 20µm thick transverse sections using a cryostat. Sections were mounted on a series of 10 positively charged slides, such that the adjacent sections mounted on each slide represented regions spaced 200µm apart.

2.2.6.2 Immunohistochemistry

Sections were double stained for glial fibrillary acidic protein (GFAP, an astrocyte marker) and NeuN (a neuronal cell body marker), or neurofilament 200 (NF200, an axonal marker) and protein zero (P₀, a peripheral myelin marker). Briefly, after blocking with 10% donkey serum in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 and 0.1% sodium azide (PBST azide) for 1 hour at room temperature (RT), the sections were incubated in PBST azide containing polyclonal rabbit anti-GFAP (1:2000, DakoCytomation) and monoclonal mouse anti-NeuN (1:500, Millipore), or monoclonal mouse anti-NF200 (Clone N52, 1:400, Sigma-Aldrich) and polyclonal chicken anti-P₀ (1:1000, ThermoScientific) overnight at RT. After 4 washes of 5 minutes in PBS,

sections were incubated in PBST azide containing donkey anti-rabbit Alexa 488 (1:1000, Invitrogen) and donkey anti-mouse Alexa 546 (1:1000, Invitrogen), or donkey anti-mouse Alexa 546 (1:1000, Invitrogen) and donkey anti-chicken Alexa 488 (1:1000, Invitrogen) for 4 hours at RT. After 4 washes of 5 minutes in PBS, sections were then coverslipped with Vectashield mounting medium (Vector laboratories). Images were taken on a Zeiss LSM 710 upright confocal microscope at the same settings and in single sessions.

2.2.6.3 Eriochrome cyanine histochemistry and tissue sparing analysis

For differentiating grey and white matter, Eriochrome Cyanine R histochemistry was performed on spinal cord sections using a protocol adapted from previously published methods (Rabchevsky et al., 2001; Springer et al., 2010). Sections were dehydrated in an ethanol series (5 minutes each), cleared in Histochoice (5 min), rehydrated in a reverse ethanol series followed by distilled water (dH₂O; 5 minutes each), then left in a solution containing 0.16% Eriochrome Cyanine R, 0.5% sulphuric acid and 0.4% iron chloride (in dH₂O) for 10 minutes to stain myelinated fibres. Following staining, sections were washed twice in dH₂O (5 minutes each), differentiated in 0.5% ammonium hydroxide (30 seconds) and washed in dH₂O (2 x 5 minutes). Finally, sections were dehydrated and cleared, as above, and then mounted using DPX. Sections were examined using a Zeiss Axioskop microscope and pictures were taken of sections at 800µm intervals throughout the lesion site at each time point (1d, 1wk, 2wk, 4wk, 12wk, 6mth; n = 4 per time point) using a Zeiss AxioCam MRm. Images were analysed using Axiovision software, which allowed the tracing of the spinal cord perimeter and the central cavity for each image captured, giving the total area for each of these measurements. The lesion epicentre was defined as the section from each animal with the largest cavity area and quantification was carried out at 800µm intervals from 3.2mm caudal to 3.2mm

rostral to the epicentre. Cavity area was calculated as a percentage of spinal cord area (i.e. area within the tracing of spinal cord perimeter) for each section. Cavity volume was then calculated over a 4.8mm segment of spinal cord, with lesion epicentre located centrally (the cavity very rarely extended beyond this distance).

2.2.6.4 Electron microscopy

Animals were terminally anaesthetised using sodium pentobarbital (Euthatal; 80 mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 3% glutaraldehyde and 4% PFA in 0.1 M PB. Immediately after perfusion a section of spinal cord was removed (~20mm) with the lesion epicentre located centrally. 3 mm sections were taken from the lesion epicentre and from the rostral and caudal lesion borders and post-fixed in the same fixative for a minimum of 48 hours at 4°C. After washing in 0.1 M PB, sections were osmicated, dehydrated in a graded ethanol series, and embedded in epoxy resin (TAAB Embedding Materials). 1µm transverse semithin spinal cord sections were cut on a microtome and stained with 0.25% toluidine blue solution before being examined on a Zeiss Axioskop light microscope in order to determine the region of the lesion epicentre to be further processed for ultrastructural analysis. For electron microscopy (EM), blocks containing the lesion epicentre were trimmed and transverse ultrathin sections were cut on an ultramicrotome and stained with lead uranyl acetate by the Centre for Ultrastructural Imaging (King's College London). Ultrathin sections were mounted on unsupported 100 mesh grids, and were visualised on a Hitachi H7600 transmission electron microscope. For analysis, photographs of the region containing the ascending dorsal column projection from each animal were taken at a magnification of 6000X. Full montages of grid squares were taken and randomly chosen images from a given grid square were analysed. For analysis of proportion of non-myelinated axons with diameters >1 µm, myelinated and non-myelinated axons were analysed from the

same randomly chosen images. For analysis of axons depending on their location relative to the cavity border, a separation was made between grid squares located furthest away or closest to the cavity border. Randomly selected images from at least two grid squares, one of which was adjacent to the cavity border and one of which was bordering the dorsal edge of the spinal cord, were then analysed and compared with respect to G ratio values and non-myelinated axons. The G ratios of approximately 500 axons and non-myelinated axon diameters of approximately 200 to 300 axons, chosen randomly, were measured per animal using AxioVision LE, release 4.2, software.

2.2.7 Statistical Analysis

An *a priori* power analysis was carried out using G*Power 3 software to estimate optimal group sizes for the primary endpoint of electrophysiological analysis. For analysis of behavioural deficits, electrophysiological percentage conduction and tissue volume a one-way ANOVA was used followed by Tukey's *post-hoc* test. Conduction velocity and cavity area measurements and comparisons of myelin at differing locations were analysed using a two-way repeated measures (RM) ANOVA followed by Bonferroni's *post-hoc* comparison. G ratio frequency distributions were analysed using a Kolmogorov-Smirnov test. All tests for statistical significance were conducted using GraphPad Prism 5. All data are presented as mean \pm standard error of the mean and $p < 0.05$ was classed as significant.

2.2.8 Additional contributions

All electron microscopy imaging and analysis was performed by Dr Katalin Bartus.

2.3 Results

2.3.1 Assessment of behavioural deficits following contusion injury

2.3.1.1 BBB locomotor rating

BBB scoring was performed to assess changes in gross locomotor performance following contusion injury (Fig. 2.4A). Prior to contusion surgery all animals achieved a baseline score of 21, indicating normal locomotion and hindlimb function. At all post-injury time points (from 1 day to 6 months) animals were substantially and significantly impaired compared to baseline values ($p < 0.001$, one-way ANOVA, Tukey's *post-hoc*). Locomotor function was most severely impaired acutely post-injury, with a mean BBB score of 3.4 ± 0.6 at 1 day post-injury (representing extensive movement of two hindlimb joints or slight movement of all three hindlimb joints). Some spontaneous recovery was observed over the initial 2 week period post-injury, with a mean BBB score of 10.2 ± 0.4 by 1 week post-injury, indicating that animals were now capable of occasional weight-supported steps, but that there was a complete lack of forelimb-hindlimb coordination. By 2 weeks post-injury the mean BBB score was 11.9 ± 0.3 , indicating that animals were now regularly taking weight-supported steps and that there was occasional forelimb-hindlimb coordination. Spontaneous recovery plateaued at this point, with BBB scores remaining at this level into chronic post-injury stages (13.1 ± 0.4 at 6 months post-injury), as although forelimb-hindlimb coordination became more frequent it was exceptionally rare for an animal to achieve consistent coordination and this limited scores to a maximum of 13.

2.3.1.2 Horizontal Ladder

Animals could not perform the horizontal ladder test until they were taking weight-supported steps and therefore, the first post-injury assessment using this test was not

until 1 week post-injury. Prior to receiving a contusion injury, animals rarely missed or slipped during hindlimb paw placement on the horizontal ladder (0.9 ± 0.2 hindlimb footslips at baseline). As with the BBB locomotor rating, animals were substantially and significantly impaired at all post-injury time points compared to the baseline values ($p < 0.001$, one-way ANOVA, Tukey's *post-hoc*; Fig. 2.4B), with the most severe deficit present at the earliest time point assessed (41.2 ± 3.6 hindlimb footslips at 1 week). Some spontaneous recovery was again observed, with improvements in performance on the horizontal ladder continuing until 4-5 weeks post-injury (20.74 ± 3.4 and 17.89 ± 2.65 footslip errors at 4 and 5 weeks, respectively). Spontaneous recovery plateaued at this point, with footslip errors remaining at this level into chronic post-injury stages (15.92 ± 3.1 at 6 months post-injury).

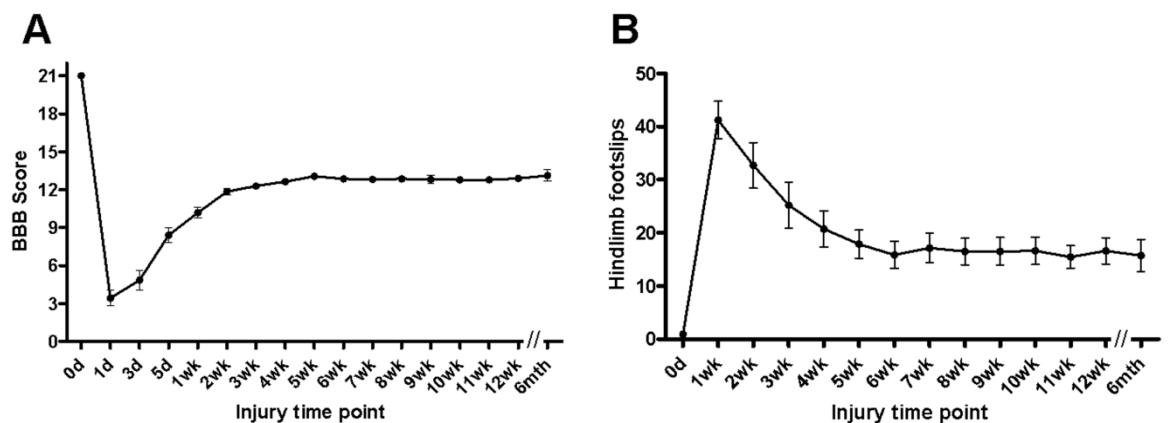


Figure 2.4. 150 kdyne contusion leads to significant and permanent behavioural deficits.

(A) BBB locomotor rating scores show a severe deficit in locomotor function at acute time points post-injury, followed by some spontaneous recovery which plateaus around 2 weeks post-injury. (B) As with BBB locomotor rating scores, the horizontal ladder test shows severe paw placement deficits at acute time points post-injury, this is followed by some spontaneous recovery which plateaus around 4-5 weeks post-injury. Error bars represent SEM.

2.3.2 Electrophysiological assessment of conduction failure following contusion injury

2.3.2.1 Conduction of the ascending dorsal column pathway

By stimulating below the injury site, activating the ascending sensory pathway antidromically and recording from dorsal root filaments, it was possible to quantify the number of functional fibres in that particular filament. Stimulating above the injury site then allowed quantification of the percentage of those fibres surviving and still capable of conduction through the lesion (Fig. 2.5A,C+D). In naïve animals all single units (activity of single nerve fibres) observed whilst stimulating with the caudally positioned electrode were still present when stimulation was switched to the rostral electrode, indicating that both electrodes were capable of maximally activating each of the filaments tested. As with behavioural assessment of function, spinal cord contusion resulted in substantial and significantly impaired function, with a dramatic reduction in the percentage of conducting fibres observed at all post-injury time points compared to naïve animals ($p < 0.001$, one-way ANOVA, Tukey's *post-hoc*; Fig. 2.5A). Acutely post-injury (1 day), there was a complete lack of conduction through the lesion site. This lack of conduction persisted at 1 week post-injury in most cases, although one animal at this time point displayed a limited amount of functionality resulting in a mean level of conduction of $1.32\% \pm 1.32$. As with behavioural function, some spontaneous improvement in conduction occurred over the sub-acute stages of injury. By 2 weeks post-injury there were a small number of fibres capable of conducting through the lesion in all animals tested ($6.5\% \pm 1.87$), which continued to increase until 4 weeks post-injury ($13.73\% \pm 1.78$); this marked a significant improvement compared to the level of conduction observed at 1 week post-injury ($p < 0.05$, one-way ANOVA, Tukey's *post-hoc*). There were no further significant improvements in conduction at later time points,

with the percentage of sampled fibres capable of conducting across a contusion injury remaining at this level into chronic post-injury stages ($16.36\% \pm 2.57$ at 6 months post-injury).

2.3.2.2 Conduction velocities of surviving fibres

One advantage of recording from a direct pathway (i.e. no synapses involved) was that it allowed an accurate calculation of the conduction velocity of the individual fibres recorded from. At every time point assessed there was a significant slowing in the conduction velocity of the fibres that remained capable of conduction through the lesion site when compared with the conduction velocity of fibres activated when stimulating caudal to the lesion (Fig. 2.5B; $p < 0.05$, two-way RM ANOVA, Bonferroni *post-hoc*). This suggested that although these fibres were capable of conduction, they were not functioning in an entirely normal manner. As with percentage conduction, conduction velocity of the spared fibres significantly improved over the first 4 weeks post-injury when compared with conduction velocities at 1 week post-injury (4.51 m/s compared to 16.38 m/s at 1 and 4 weeks, respectively; $p < 0.001$, two-way RM ANOVA, Bonferroni *post-hoc*). There were no further increases in conduction velocity of fibres conducting through the lesion at chronic post-injury stages (12 weeks, 6 months).

2.3.2.3 Enhanced conduction following cooling

Cooling of nerve fibres can lower their threshold for activation due to differences in the temperature sensitivities of sodium and potassium channels responsible for action potential propagation. Previously this technique has been used to restore conduction across regions of focal demyelination (Smith et al., 2000); here it was used to test the hypothesis that there may be some fibres that had survived through the lesion site but that were not capable of conduction under normal physiological conditions. Whilst the use of this technique had no effect on the number of fibres conducting in naïve

(uninjured) spinal cords, there were numerous examples of at least one single unit being added to the recording from a dorsal root filament following cooling of the lesion site at all post-injury time points, excluding 1 day post-injury (Fig. 2.5E,F), with the addition of at least one single unit observed in 10 of 22 filaments at 1 week post-injury, 4 of 20 at 2 weeks, 7 of 26 at 4 weeks, 6 of 25 at 12 weeks, and 5 of 28 at 6 months. Thus, there were viable, non-conducting axons present in approximately 20% of the filaments tested at all post-injury time points other than 1 day post-injury.

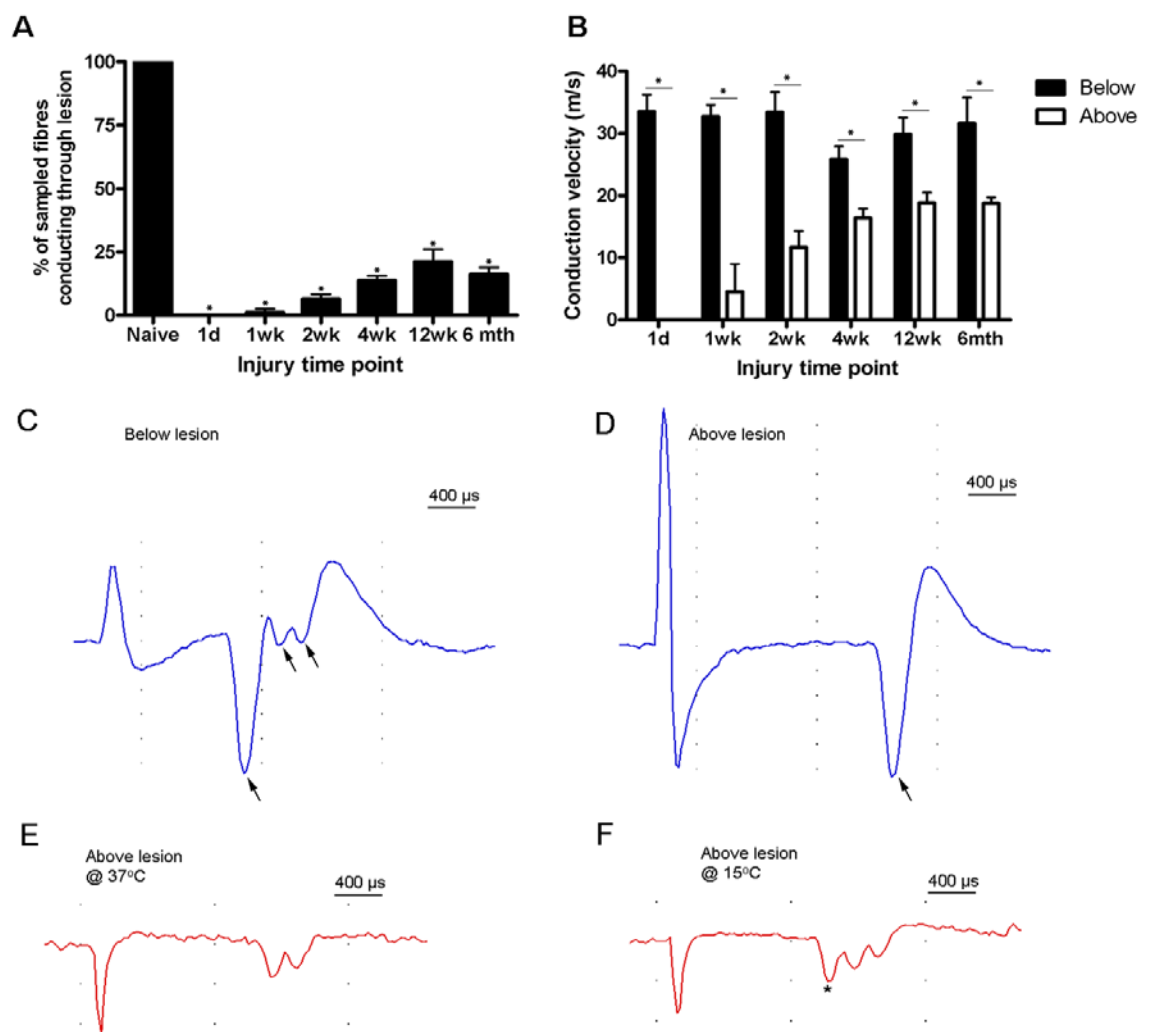


Figure 2.5. 150 kdyne contusion leads to a substantial decrease in the percentage of fibres capable of conducting across a contusion injury and a slowing of their conduction velocity.

(A) Functionality of the ascending sensory pathway is initially abolished post-injury, with a

complete lack of conduction acutely (1 day), followed by a partial recovery of conduction over the sub-acute phase (with ~14 % of sampled fibres capable of conducting across the contusion injury at four weeks); no further improvements in conduction were observed, with the functionality of the dorsal column pathway remaining severely impaired at the chronic post-injury time points (~16% conduction at chronic stages of spinal cord injury). **(B)** Conduction velocity measurements of individual fibres capable of conducting through the lesion site (above injury recordings) revealed a significant slowing of conduction velocity at every post-injury time point, compared to the conduction velocity of fibres activated below the injury (below injury recordings), where this tract would be relatively intact (although, as with the % conduction measurements, there was some recovery in conduction velocity over the sub-acute stages of injury). Representative traces recorded whilst stimulating below **(C)** and then above **(D)** the injury site at 12 weeks post-injury highlight the differences in conduction properties between the “intact” and injured pathway. Three single units are present whilst stimulating below the injury, but only one survives to conduct through the lesion. The delayed latency of the unit conducting through the lesion indicates impaired conduction velocity. Single units of activity (arrows) represent the activity of single nerve fibres. **(E)** and **(F)** show representative traces recorded at 12 weeks post-injury whilst stimulating above the injury and the cooling technique was applied. By cooling the lesion site with cold mineral oil additional conducting fibres could be recruited at every post-injury time point (asterisk in **F** highlights a single unit added following cooling), thus revealing an important population of axons that are viable but unable to conduct under normal physiological conditions. Error bars represent SEM; asterisks in **(A)** denote significantly impaired conduction compared to uninjured (naive) spinal cord ($p < 0.001$, one-way ANOVA, Tukey’s *post-hoc*); asterisks in **(B)** denote significantly impaired conduction velocity of dorsal column axons recorded above, compared to below, the injury ($p < 0.01$, two-way RM ANOVA, Bonferroni *post-hoc*).

2.3.3 Correlations between behaviour and electrophysiology

Since the two behavioural tests used showed a different recovery time course to one another, we correlated the behavioural scores of individual animals with the extent of conduction observed through their injury site. BBB scores showed a low positive correlation with the percentage of conducting fibres ($R^2 = 0.34$), such that improved conduction through the injury did not always correspond with higher BBB scores; this was particularly apparent over the sub-acute phase where conduction is still improving but BBB scores have already reached a plateau (Fig. 2.6A). In contrast, there was a high negative correlation between footslip errors on the ladder task and percentage fibre conduction ($R^2 = 0.63$), such that improved conduction through the injury site typically corresponded with fewer footslip errors on the horizontal ladder (Fig. 2.6B). Thus, the horizontal ladder task may be a more sensitive test for revealing subtle changes in function in this injury model.

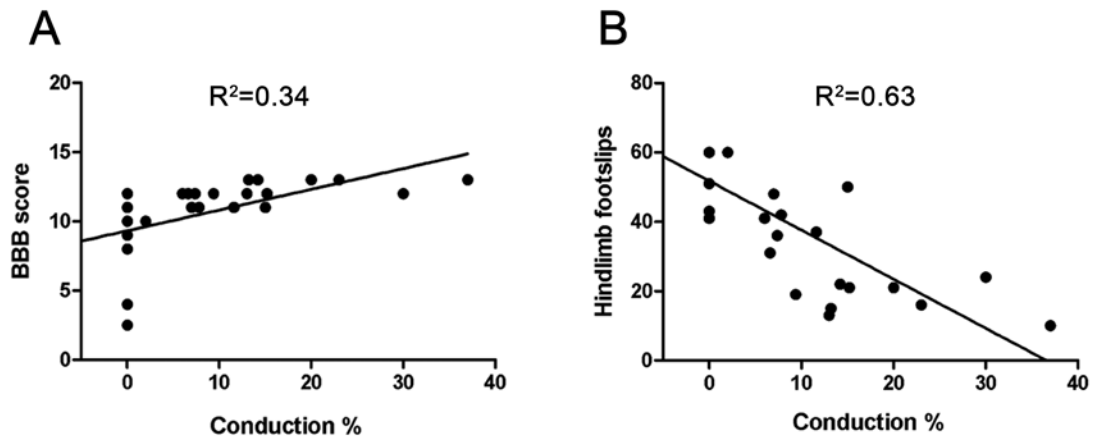


Figure 2.6. Correlations of behavioural and electrophysiological data. Statistical analysis revealed a low correlation between BBB scores and percentage conduction (**A**) and a high correlation between footslip errors and percentage conduction (**B**), where animals with improved conduction typically made fewer footslip errors on the horizontal ladder, indicating that this may be a more sensitive test than BBB locomotor rating for revealing subtle changes in behavioural function (R^2 value calculated using Pearson's correlation test).

2.3.4 Ultrastructural assessment of demyelination and remyelination following contusion injury

2.3.4.1 Electron microscopy

The dorsal column region containing the fasciculus gracilis was chosen for electron microscopic analysis as it contained the axons from which electrophysiological recordings were carried out (Fig. 2.7A-D). Dorsal column axons from naïve (uninjured) spinal cords were normal in appearance (Fig. 2.8A), while numerous axons either lacking myelin sheaths or with disrupted and unhealthy myelin were observed at 1 week post-injury (Fig. 2.8B). By 4 weeks post-injury many axons appeared to be very thinly

myelinated and often remyelination had been performed by Schwann cells, identified by the presence of a basal lamina and the characteristic features of Schwann cell nuclei (Fig. 2.8C), suggesting that remyelination was taking place and was often mediated by Schwann cells. At a more chronic post-injury time point (12 weeks) many axons now had dense, healthy myelin sheaths and, again, this was often associated with Schwann cells (Fig. 2.8D). There was a significant increase in the mean G-ratio at 1 week (mean G ratio = 0.77 ± 0.012) and 4 weeks (mean G ratio = 0.79 ± 0.01), compared to axons in uninjured spinal cords (mean G ratio = 0.64 ± 0.023 ; $p < 0.02$, One-way ANOVA, Tukey's *post-hoc*), with a significant shift in G ratio frequency distribution ($p < 0.0001$, Kolmogorov-Smirnov test; Fig 2.8E). This shift had reversed by 12 weeks post-injury, indicating that most axons had comparable, or thicker, myelin sheaths to those observed in uninjured spinal cord (Fig. 2.8E), which presumably is a consequence of Schwann cell remyelination. No significant changes in axon calibre of myelinated axons were found at any of the time points assessed. Additionally, while in uninjured spinal cords only axons with a diameter of $\leq 1 \mu\text{m}$ were non-myelinated, significant populations of non-myelinated large-diameter axons ($> 1 \mu\text{m}$) were present following contusion injury at all post-injury time points ($p < 0.001$, one-way ANOVA, Tukey's *post-hoc* test; Fig 2.8F). Thus, at all post-injury time points there remained a proportion of axons that were demyelinated following injury and had not been remyelinated.

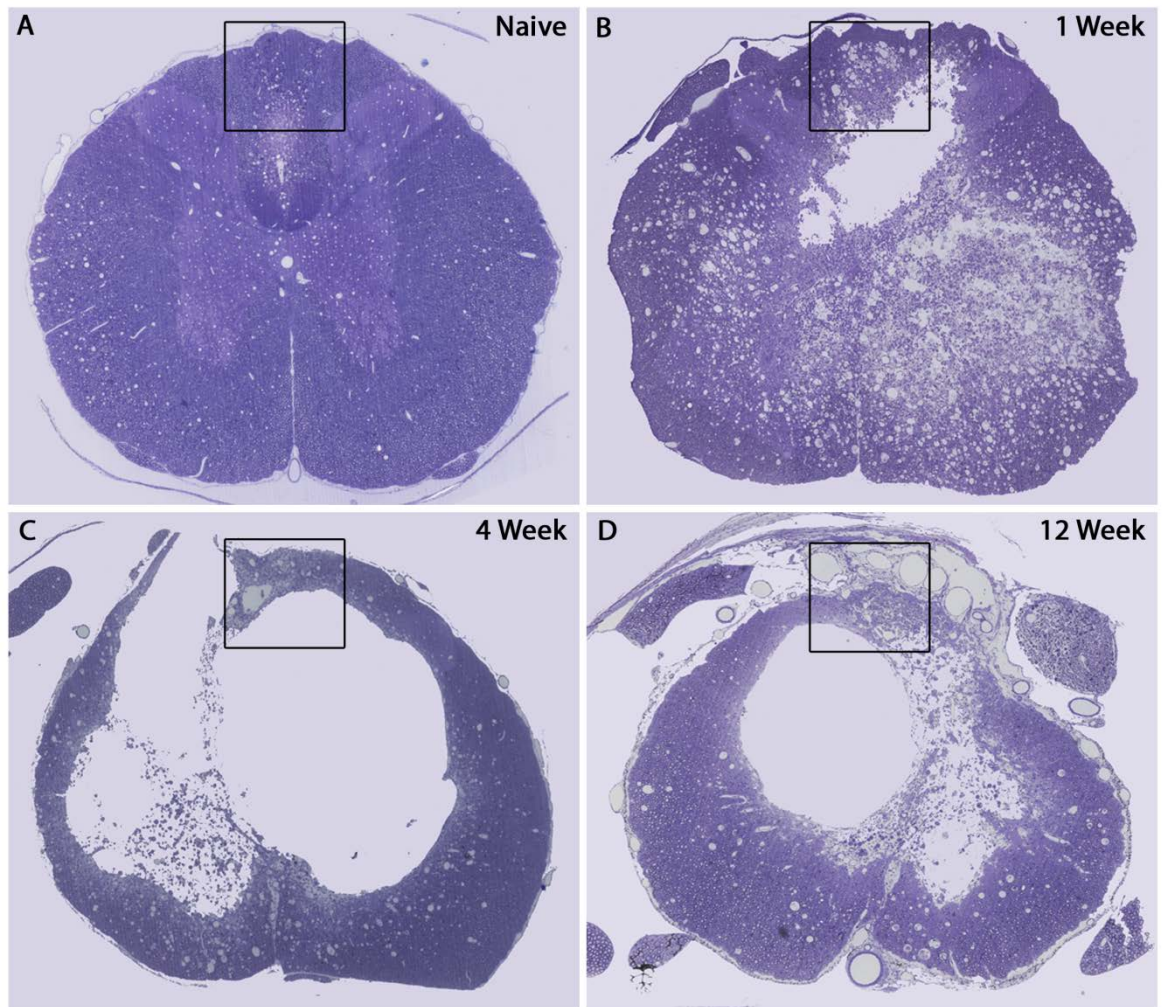


Figure 2.7. Transverse semithin spinal cord sections stained for Toluidine blue. Images taken from an uninjured (naïve) spinal cord (A) or from the lesion epicentre at 1 week (B), 4 weeks (C) and 12 weeks (D) after contusion injury. The boxed areas indicate the regions processed for electronmicroscopy, containing the ascending dorsal column axons, and correspond to the electron micrographs in Figure 2.8 A-D.

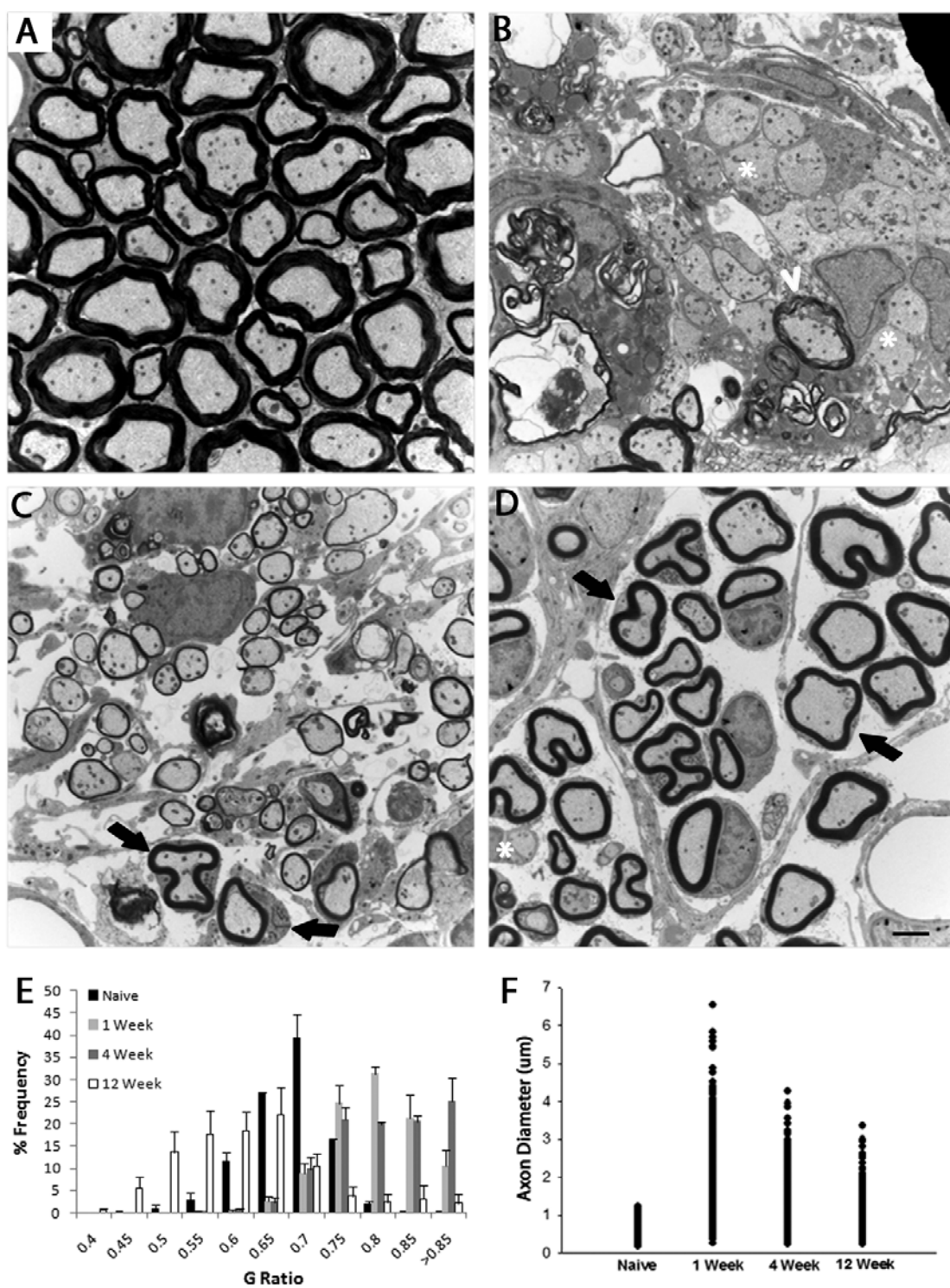


Figure 2.8. Time course of demyelination and subsequent remyelination of dorsal column axons at the contusion lesion epicentre. (A) Electron micrograph of dorsal column axons from an uninjured (naïve) spinal cord illustrating healthy myelin sheaths. (B) At 1 week post-injury extensive demyelination is apparent (asterisks) and remaining myelin appears unhealthy as it becomes less compact and often appears to be unravelling (white arrow head). (C) By 4 weeks post-injury some remyelination is apparent and both non-myelinated and thinly remyelinated axons are found throughout the dorsal columns. Some axons associated with Schwann cells are also apparent (black arrows). (D) Remyelination progresses and at 12 weeks post-injury many axons have myelin sheaths that are thick and compact in appearance and are often associated with Schwann cells (black arrows). (E) G ratio frequency distributions show a significant shift towards high proportions of thinly myelinated axons at 1 and 4 weeks post-injury compared to naive (uninjured) dorsal column axons ($p < 0.0001$, Kolmogorov-Smirnov test), with this shift reversed by 12 weeks post-injury. (F) Graphic plot displaying numbers and diameter of non-myelinated axons measured at each time point shows that significant populations of large-diameter axons ($>1 \mu\text{m}$) that lacked myelin sheaths were present following contusion injury at all post-injury time points, compared to uninjured spinal cords where only axons with a diameter of $\leq 1 \mu\text{m}$ were non-myelinated ($p < 0.001$, one-way ANOVA, Tukey's *post-hoc* test). Scale bar, $2 \mu\text{m}$.

Analysis was carried out to determine whether there were any differences in myelin sheath thickness and/or proportion of large-diameter ($>1\ \mu\text{m}$) non-myelinated axons depending on the location of the axons relative to the cavity border. Dorsal column axons located furthest away from the cavity border were compared to axons closest to the cavity (Fig. 2.9F). G ratio quantification revealed no difference between these two locations in the uninjured spinal cord ($p>0.05$, two-way RM ANOVA; Fig. 2.9A) or at 12 weeks post-injury ($p>0.05$, two-way RM ANOVA; Fig. 2.9D). In contrast, at 1 week (Fig. 2.9B) and 4 weeks (Fig. 2.9C) post-injury, there was a significant increase in the mean G ratio of axons close to the cavity border compared to axons located in the most dorsal regions of the dorsal columns (mean G ratio for 1 week post-injury = 0.80 ± 0.01 and 0.76 ± 0.02 for closest to and furthest away from cavity, respectively; mean G ratio for 4 weeks post-injury = 0.82 ± 0.01 and 0.76 ± 0.01 for closest to and furthest away from cavity, respectively; $p<0.05$, $p<0.001$ for frequency distribution dorsal vs ventral location at 1 and 4 weeks post-injury, respectively, two-way RM ANOVA, Bonferroni *post-hoc*). Similarly, there were significantly more large-diameter ($>1\ \mu\text{m}$) non-myelinated axons observed closest to the cavity border at 1 week and 4 weeks post-injury ($p<0.05$ and $p<0.001$ for dorsal vs ventral location at 1 and 4 weeks, respectively; two-way RM ANOVA, Bonferroni *post-hoc* Fig. 2.9E). A similar trend was apparent at 12 weeks post-injury, but did not reach statistical significance.

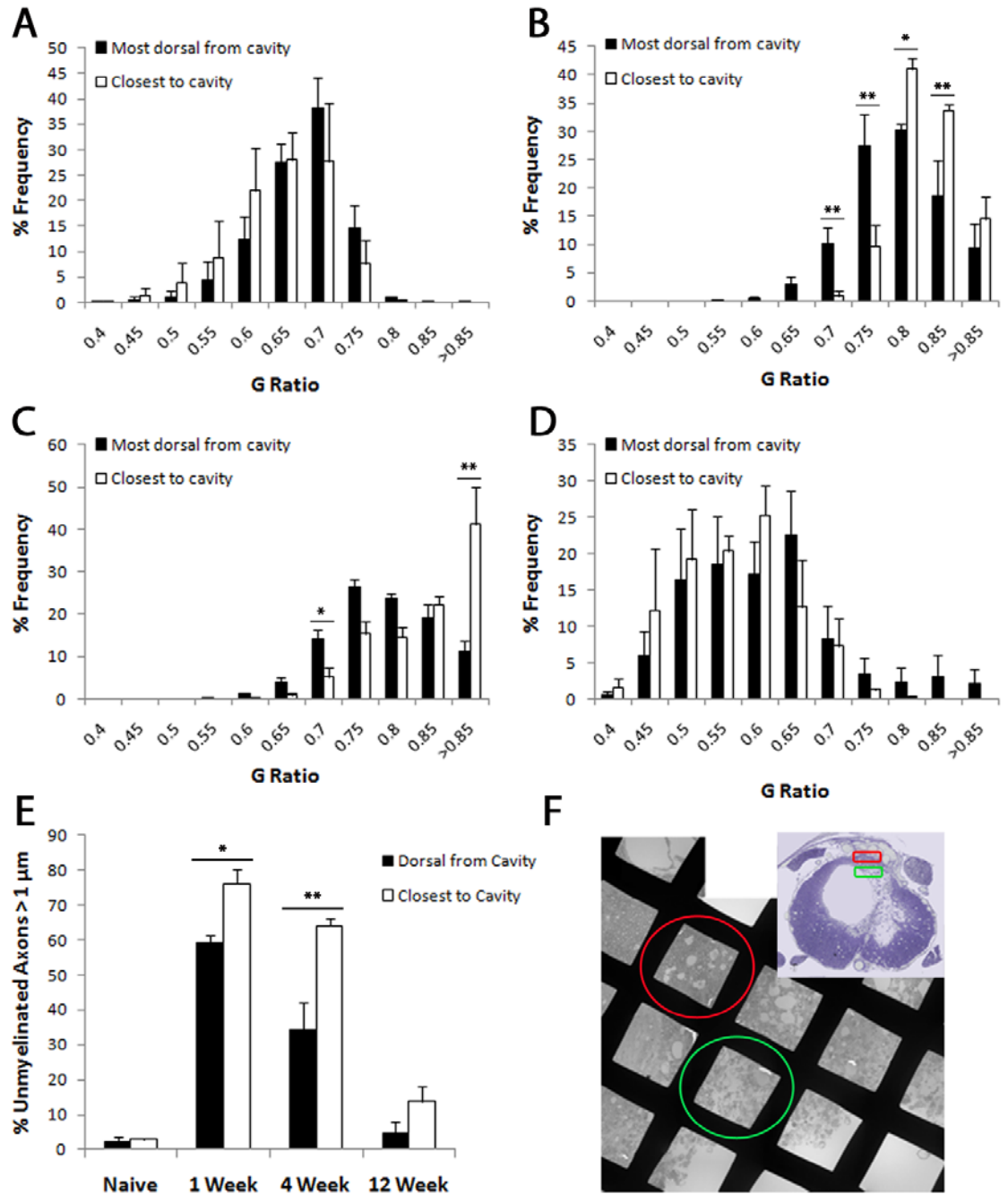


Figure 2.9. Comparison of the state of myelination of dorsal column axons located either furthest away from the cavity edge (dorsal from cavity) or adjacent to the cavity (closest to cavity). (A-D). G ratio frequency distributions reveal no differences in myelin sheath thickness in the two regions in uninjured (naïve) spinal cords (A) or 12 weeks post-injury (D). However, at 1 week (B) and 4 weeks (C) post-injury many more thinly myelinated axons are located close to the cavity border while axons with thicker myelin sheaths at these post-injury time points are

predominantly found away from the cavity edge (* $p < 0.05$; ** $p < 0.001$; two-way RM ANOVA, Bonferroni *post-hoc*). (E) Measurements of the proportion of non-myelinated axons with diameters greater than 1 μm relative to their location from the cavity border reveals that at 1 week and 4 week post-injury significantly more non-myelinated axons are found close to the cavity border (* $p < 0.05$; ** $p < 0.001$; two-way RM ANOVA, Bonferroni *post-hoc*). (F) Illustration of compared locations of axons relative to the cavity border. Axons furthest away from the cavity border (red) were compared to axons located close to the cavity (green).

2.3.5 Schwannosis in the dorsal columns following contusion injury

Schwannosis is frequently observed in the chronically injured human spinal cord (Bruce et al., 2000; Guest et al., 2005) where, as a result of Schwann cell invasion and/or proliferation, central axons become associated with peripheral myelin. Co-localisation of the neurofilament marker NF200 with the Schwann cell marker P_0 at the lesion epicentre revealed positive staining for peripheral myelin around numerous dorsal column axons at 4 weeks (Fig 2.10B) and 12 weeks (Fig. 2.10C-E) following contusion injury. NF200 and P_0 co-localisation in the dorsal columns was not apparent at earlier (1 week) stages post-injury (Fig. 2.10A). These findings are in agreement with the ultrastructural observations and indicate that, as the injury progresses into the chronic stages, Schwann cells present in the dorsal columns following contusion injury remyelinate demyelinated axons.

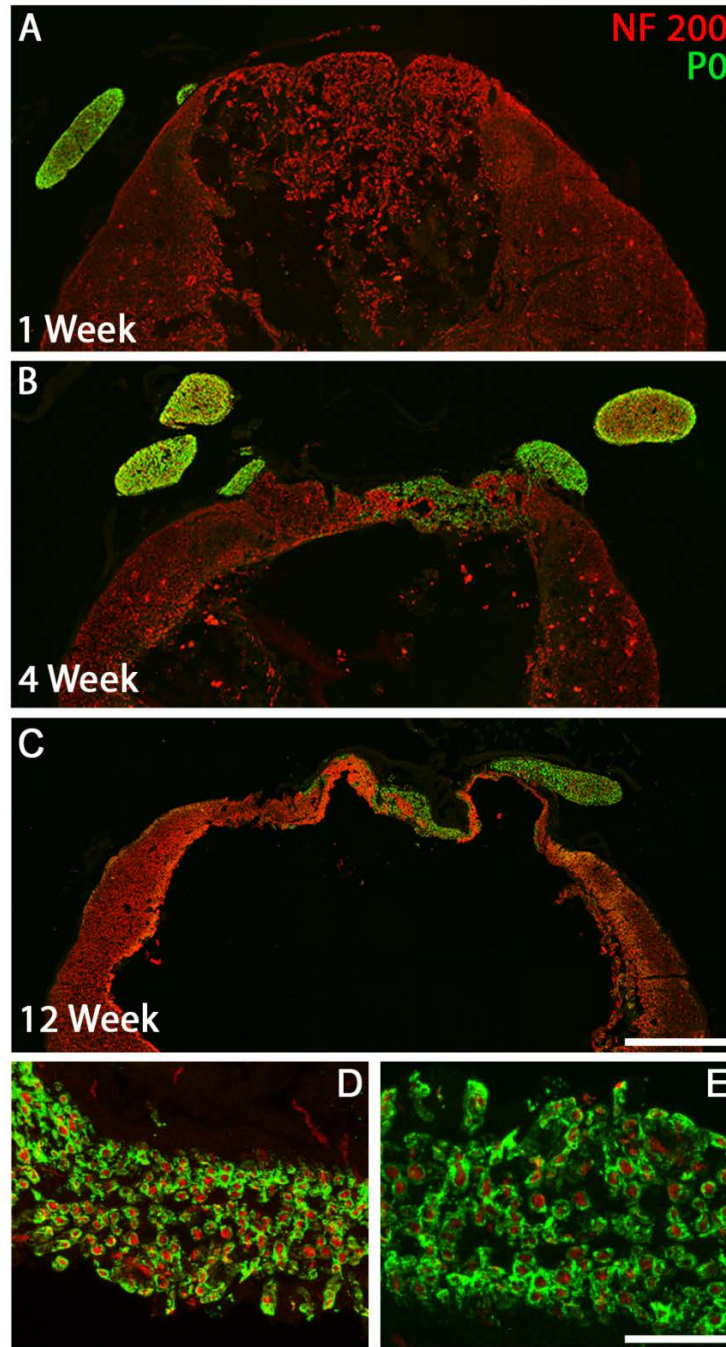


Figure 2.10. Remyelination of dorsal column axons at the lesion epicentre is frequently associated with Schwann cells. Co-staining of axons (NF 200, red) and Schwann cell associated myelin (P₀, green) illustrates the presence of remyelinating Schwann cells in the dorsal column of the spinal cord at the lesion epicentre at 4 and 12 weeks post-injury (**B,C**) but not at 1 week post-injury (**A**). Images taken at higher magnification (63X oil) from the 12 week animal shown in **C** illustrate P₀ positive myelin fully ensheathing NF200 positive axons (**D,E**). Scale bar (**A-C**) = 500µm, (**D,E**) = 20µm.

2.3.6 Histological assessment of lesion pathology following contusion injury

2.3.6.1 Cavity formation, reactive gliosis and neuronal cell loss

Co-staining of transverse sections with NeuN (a neuronal cell marker) and GFAP (an astrocyte marker) revealed a number of progressive pathological changes that occur over time following contusion injury. At the lesion epicentre these were characterised by a loss of neuronal cell bodies, mass destruction of spinal cord grey matter, increasing cavity formation and reactive gliosis (Fig. 2.11A-F). Acutely (1 day) post-injury, marked necrosis of the grey matter was already apparent, with very few NeuN-positive neuronal cell bodies remaining at the lesion epicentre (Fig. 2.11B); this progressed over the sub-acute (1-2 weeks; Fig. 2.11C+D) to chronic (4-12 weeks; Fig. 2.11E+F) stages, with a complete absence of NeuN-positive cell bodies from 2 weeks post-injury and necrosed tissue areas giving rise to chronic central cavities. Co-staining with GFAP showed a dramatic increase in reactive astrocytosis throughout the lesion epicentre at the acute stage (Fig. 2.11B). As the injury progressed, the reactive gliosis appeared to become more focussed on the perimeter of the developing cavities, forming a dense glial scar around them (Fig. 2.11C-F). Differentiation of grey and white matter using eriochrome cyanine R histochemistry highlighted the extent and progression of grey and white matter loss from acute to chronic stages following contusion injury, with debris and necrosed tissue in the central core of the lesion at early post-injury stages being gradually cleared to leave behind a large central cavity, surrounded by a rim of spared white matter (Fig. 2.11G-L).

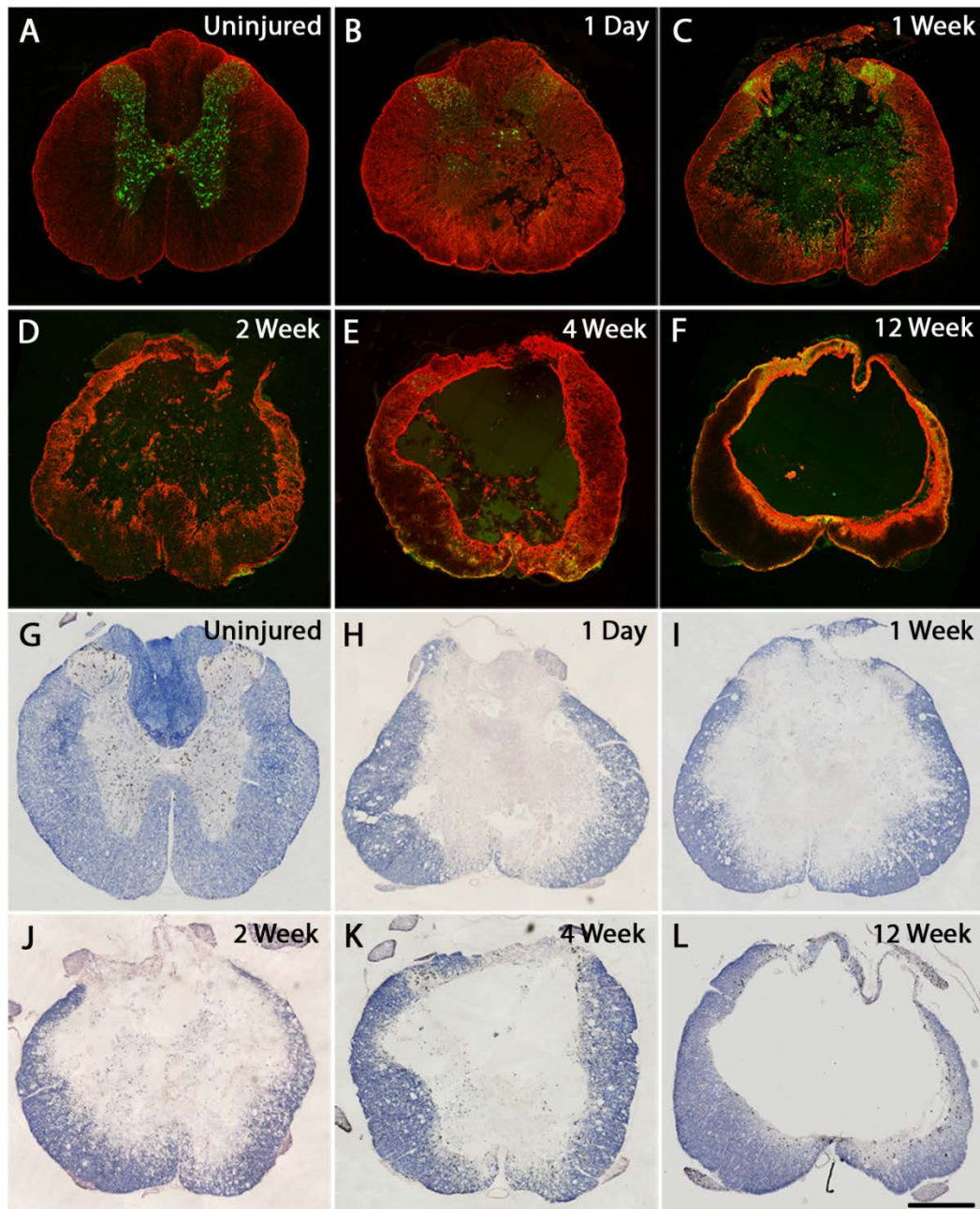


Figure 2.11. 150 kdyne contusion leads to progressive cell loss, reactive gliosis and cavitation. GFAP and NeuN immunohistochemistry (A–F) and eriochrome cyanine staining (G–L) in transverse sections of the spinal cord through epicentre of the injury demonstrate the pathological changes taking place from acute to chronic time points post-injury (1 day–12 weeks) and can be compared to uninjured spinal cord (A+G). GFAP (astrocytes, red) and NeuN (neuronal cell bodies, green) co-staining illustrates the progressive destruction of the grey matter and neuronal cell loss that occurs following injury as well as the pronounced increase in

reactive gliosis acutely following injury (**B**), which at later stages becomes more localised to the borders of the cavity, forming a dense glial scar (**C-F**). Staining with eriochrome cyanine (to demarcate grey and white matter areas) highlights the initial phase of mass necrosis in the spinal parenchyma at early stages (**H,I,J**) followed by clearance of the debris, resulting in large central cavities surrounded by a spared rim of white matter at chronic post-injury time points (**K,L**). Scale bar = 500µm.

2.3.6.2 Tissue sparing

In order to quantify the extent of cavity formation at the lesion epicentre as well as the rostro-caudal spread of the injury, analysis was carried out on serial sections stained with eriochrome cyanine (Fig. 2.12). The lesion epicentre was classified as the tissue section in which cavity area was largest. At each time point there was severe pathology apparent in the lesion epicentre, where mass destruction of both grey and white matter occurred; at rostro-caudal distances from the epicentre the areas of necrosis and cavitation were predominantly confined to the dorsal columns (Fig 2.12A). Quantification revealed that at acute and sub-acute time points (1 day–2 weeks), the cavity area at the lesion epicentre was approximately 10% of the total cord area. The cavity area then dramatically increased at the later post-injury time points (~25%, 30% and 40% of the total cord area at 4 weeks, 12 weeks and 6 months post-injury, respectively; Fig 2.12B). At all chronic time points (4 week, 12 week and 6 month) there was a significant increase in cavity area at the lesion epicentre in comparison to all acute and sub-acute time points (1 day, 1 week and 2 weeks; $p < 0.05$, two-way RM ANOVA, Bonferroni *post-hoc*). This is consistent with the previous observation that prior to 4 weeks the lesion core is filled with necrosed tissue and debris, which then gives rise to a central cavity. The rostrocaudal spread of the lesion also progressively

increased over this period, with greater cavity areas observed at sites increasingly distal to the lesion epicentre over time (Fig. 2.12A+B). Measurements of total cavity volume at each time point confirmed a progressive increase from sub-acute (2 weeks) to chronic (6 months) time points in the total area through the lesion that contained cavities (Fig 11C). As with cavity area, total cavity volume at chronic time points was significantly higher than that observed at acute and sub-acute time points ($p < 0.01$, one-way ANOVA, Tukey's *post-hoc*).

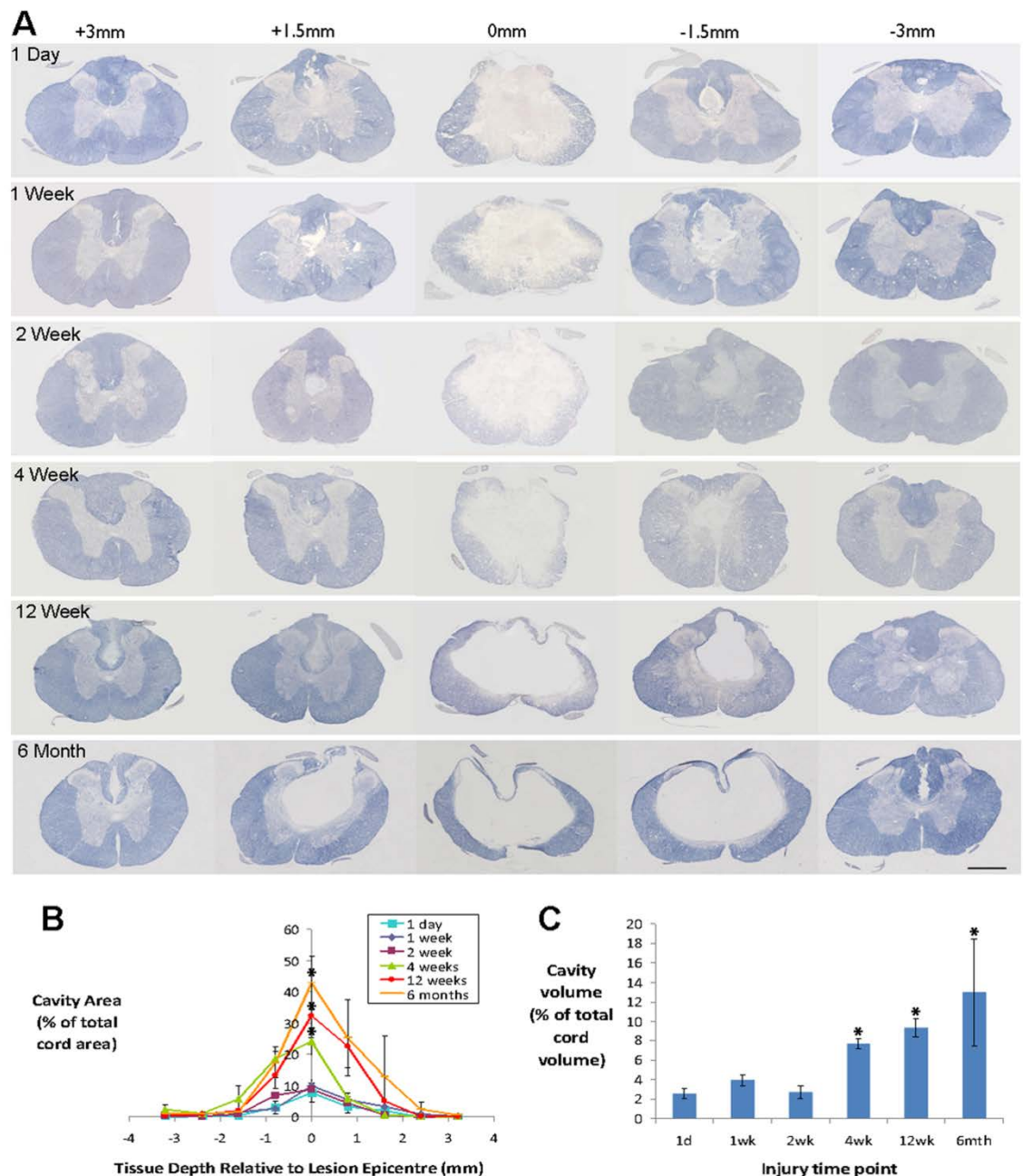


Figure 2.12. Cavity formation and rostro-caudal degeneration gradually increases over time following spinal contusion. (A) Eriochrome cyanine staining of serial tissue sections from 3 mm rostral to 3 mm caudal to the lesion site (0mm indicates the lesion epicentre) at a number of post-injury time points shows stereotypical tissue degeneration and progressive cavity formation; mass destruction of both white and grey matter is apparent at the lesion epicentre at all post-injury time points; rostro-caudal degeneration was mainly restricted to the dorsal columns at early post-injury time points, with increasing rostro-caudal cavitation observed at

later stages. **(B)** Quantification of cavity area (expressed as percentage of spinal cord area) at 800 μm intervals through the extent of the injury confirms significantly increased cavity size and rostro-caudal degeneration in the later stages of injury (from 4 weeks). **(C)** Similarly, quantification of total lesion volume, expressed as percentage of total spinal cord volume through the same section of spinal cord shows a significantly increased lesion volume at all chronic injury time-points (from 4 weeks onwards), compared to the earlier post-injury time-points (up to 2 weeks). Error bars represent SEM; asterisks denote significantly increased cavity area ($p < 0.05$, two-way RM ANOVA, Bonferroni *post-hoc*) and cavity volume ($p < 0.01$, one-way ANOVA, Tukey's *post-hoc*) at all chronic injury time-points (4 weeks, 12 weeks and 6 months), compared to acute and sub-acute injury time-points (1 day, 1 week, 2 weeks). Scale bar = 500 μm .

2.4 Discussion

The present study demonstrates for the first time the temporal pattern of conduction failure across a contusion injury and changes in the conduction properties of individual fibres over an acute to chronic time course in a clinically relevant model of SCI. A combination of techniques was utilised to carry out detailed assessments of the functional and pathological changes that occur post-injury. Established behavioural techniques depicted the time course of gross changes in function post-injury, highlighting the spontaneous recovery that occurs over sub-acute time points, but that substantial deficits remain into the most chronic time points. The application of a novel electrophysiological technique revealed more detailed information about changes in the functionality of a specific spinal pathway, showing complete conduction failure of ascending dorsal column axons at acute time points followed by some spontaneous recovery, and that the fibres in which conduction was restored did so at a significantly decreased conduction velocity. Furthermore, a population of axons that survive the SCI, but are unable to conduct under normal physiological conditions was identified. By applying a cooling technique, previously used in demyelination injury studies, it was demonstrated that conduction across a contusion injury can be restored to these axons, even in the chronically injured spinal cord. EM analysis revealed the temporal pattern of demyelination and subsequent remyelination after SCI, with a time course that corresponded to functional changes, and also revealed a population of chronically demyelinated axons which are likely to be the population of axons that responded to cooling with restored conduction. Thus, these studies provide mechanistic insight into functional changes that occur following SCI and identify an important population of

axons that are chronically functionally compromised but remain accessible to strategies to restore conduction.

2.4.1 Chronic conduction failure and changes in conduction properties following spinal contusion

2.4.1.1 Temporal changes in extent of conduction failure

The immediate loss of conduction at 1 day post-injury is likely due to the effects of spinal shock (Atkinson and Atkinson, 1996;Smith and Jeffery, 2005;Bradbury and McMahon, 2006), a process which is thought to be caused by a number of contributing factors such as physical damage to axons, local haemorrhage at the injury site and ischaemia due to vascular injuries (Tator and Fehlings, 1991;Tator, 1991;Dumont et al., 2001;Ditunno et al., 2004). Haemorrhage could lead to the release of molecules that are inhibitory to conduction, for example nitric oxide (NO) levels are increased at the site of SCI (Sharma et al., 1996;Winkler et al., 1998) and NO has previously been shown to block conduction (Redford et al., 1997). The effect of the injury on supporting cells, particularly oligodendrocytes, may also play a role in the lack of conduction at 1 week post-injury since previous work has shown that contusion injury leads to damage and apoptosis of myelinating oligodendrocytes (Shuman et al., 1997), resulting in focal demyelination. Demyelination is known to result in conduction block, having been demonstrated electrophysiologically in models of focal demyelination induced by injection of diphtheria toxin in cats (McDonald and Sears, 1969a;McDonald and Sears, 1970a) as well as focal demyelination induced by injection of ethidium bromide in rats (Smith et al., 2000). EM analysis at 1 week post-injury indicated the presence of completely demyelinated axons as well as unhealthy non-compacted myelin, suggesting

that demyelination was taking place at this time point and was an important contributing factor to the loss of conduction. Conversely, as cell death and axonal degeneration progress there are also intrinsic repair mechanisms that occur following SCI, reflected here by the gradual increase in conductivity at 2 and 4 weeks post-injury. This finding supports previous anatomical observations in a rat thoracic contusion injury model which suggested that many axons which were damaged by the initial trauma, but survived, are likely to have undergone gradual repair as they appeared less swollen at later time points with healthier myelin sheaths (Beattie et al., 1997b). Whilst the findings of this study related to the anatomical status of surviving axons, the anatomical repair observed may well have been associated with restoration of conductivity as indicated by the findings of this chapter. In addition, EM analysis at 4 weeks post-injury indicated that many axons have become thinly remyelinated by this time point; this is likely to be sufficient to restore conduction in these axons, provided they are otherwise intact. Any initial conduction block caused by NO would now be alleviated due to repair of the vasculature and a decrease in the levels of NO at the injury site (Nakahara et al., 2002). The recent finding that acute application of CSPGs can block axonal conduction in the spinal cord suggests that these molecules may also play a role in the changes in conductivity post-injury (Hunanyan et al., 2010). This study hypothesised that the diminished conduction of intact fibres contralateral to a spinal hemisection was partially due to the associated upregulation of CSPGs, showing that ChABC treatment of a unilateral hemisection injury could prevent this loss of conduction in spared ventrolateral funiculus axons. In an acute experiment, intraspinal injection of the CSPG NG2 was then shown to depress axonal conduction through the injection site in a dose dependent manner, whilst injection of saline had no effect on conduction (Hunanyan et al., 2010). These findings have been further substantiated by a more recent study

indicating that the CSPG NG2 is particularly potent in blocking axonal conduction (Petrosyan et al., 2013). In this study the application of an antibody against NG2 was shown to restore conduction to axons in which conduction had been acutely blocked by application of NG2 as well as in spared axons following a spinal hemisection injury (Petrosyan et al., 2013). CSPGs are known to be highly up-regulated acutely post-injury (Lemons et al., 1999) and CSPG expression at the injury site persists at chronic injury time points (Tang et al., 2003; Jones et al., 2003a; Iaci et al., 2007). CSPG expression could therefore, provide a key mechanism for the persistent conduction failure observed at all time points post-injury.

2.4.1.2 Temporal changes in conduction velocity

Significant deficits in conduction velocity in the small proportion of axons that were able to conduct across the contusion site were observed at all post-injury time points. Many of the mechanisms responsible for changes in conduction levels may also contribute to the observed changes in conduction velocity. Damaged axons undergoing repair may be capable of action potential propagation, but an incomplete state of repair could weaken conduction due to processes such as ion leakage and inefficient insulation of the axon. Some focally demyelinated fibres may be capable of conduction, but conduction across the demyelinated section of the axon will be severely slowed (McDonald and Sears, 1970b). The remyelination observed 4 weeks post-injury in the present study is likely to contribute to the recovery in conduction velocity by this time point. However, despite significant remyelination taking place, conduction velocities remain significantly impaired at 6 months post-injury. The impairment in conduction could be caused by properties of the newly formed myelin itself, particularly as the remyelination is shown here to be primarily mediated by Schwann cells, which is likely to have different properties to central myelin, such as the density of the myelin and the

level of electrical insulation it provides. The average internodal length of newly formed myelin following some demyelinating lesions is significantly shorter than normal (Blakemore and Murray, 1981); if this is mirrored in the remyelination seen in the present study, this could also contribute to the impaired conduction velocity.

2.4.1.3 Novel population of viable but non-conducting axons

Whilst this is the first time that conduction changes in individual fibres have been assessed *in vivo* following spinal contusion in the adult rat, a number of previous studies have examined motor or sensory evoked potentials following contusion, often using non-invasive magnetic stimulation paradigms and/or minimally invasive needle electrodes, to provide information regarding the effects of contusion on complete tracts in the spinal cord (Khan et al., 1999; Metz et al., 2000a; Cao et al., 2005b; Beaumont et al., 2006; Ellingson et al., 2008). As in the present investigation, these studies showed a substantial decrease in amplitude of response post-injury, often followed by a degree of spontaneous recovery at later time points. In instances where dorsal column functionality has been assessed at chronic time points, amplitudes of potentials were reduced to 10-20% of pre-injury levels (Ellingson et al., 2008), in agreement with the percentage of fibres found to be conducting through a contusion injury at the most chronic time point in the present study (~ 14 % at 6 months post-injury). Hains et al (2004) used more invasive methods to look in more detail at conduction of sensory axons across a contusion injury by stimulating the lumbar dorsal columns and recording compound action potentials above the contusion injury, to assess protective effects of a sodium channel blockade on conduction of the dorsal column pathway one month after injury. This study also found that conduction in the dorsal columns was reduced to ~15% of pre-injury levels. In the present study recordings were made from individual fibres teased from dorsal root filaments to obtain detailed information on spared fibres

and thus reveal important information about the integrity of the fibre, such as whether it is damaged or demyelinated. Conduction properties of sensory fibres have previously been examined in isolated spinal cord segments following compression injury (Blight, 1983; Nashmi and Fehlings, 2001) and these *ex-vivo* techniques have been used to demonstrate the role of voltage gated potassium channels in chronic axonal dysfunction (Nashmi et al., 2000). The present study supports previous observations on the loss and recovery of evoked potentials following thoracic and cervical contusion injury (Nashmi et al., 1997; Onifer et al., 2007) and also suggests that demyelination is a major factor in chronic conduction failure, since a key finding was that lowering the activation threshold of fibres by cooling the lesion site revealed the presence of viable nerve fibres that are unable to conduct under normal physiological conditions. Studies of primary demyelinating lesions have used a similar cooling paradigm to successfully restore conduction in some fibres (Rasminsky, 1973; Bostock et al., 1978; Smith et al., 2000), but this technique has not previously been applied to a SCI study. Cooling of the lesion prolongs action potential duration due to differences in the temperature sensitivity of sodium and potassium channels, increasing the likelihood of action potential propagation over long distances if myelination is focally impaired (Bostock et al., 1978). Thus, the findings of this chapter provide robust evidence of a population of spared axons that remain chronically compromised and unable to conduct following SCI and, importantly, that they remain accessible to strategies to restore conduction and that this can have a significant functional effect.

2.4.2 Chronic demyelination following spinal contusion

Whether demyelination persists in chronic SCIs and, if so, whether it occurs significantly enough to affect function, remains controversial in the field. Conflicting

reports have appeared from both human and experimental studies, where some reports claim little or no evidence of chronic demyelination (Kakulas, 1999;Lasiene et al., 2008;Salazar et al., 2010;Powers et al., 2012), while others report significant progressive chronic demyelination (Waxman, 1989;Bunge et al., 1993;Guest et al., 2005;Totoiu and Keirstead, 2005). Strategies to enhance remyelination have been shown to facilitate functional recovery following SCI if administered during the acute or sub-acute post-injury phase (Keirstead et al., 2005;Karimi-Abdolrezaee et al., 2006;Cao et al., 2010). In contrast, when these strategies have been applied to chronic injuries they have often had no functional benefit (Keirstead et al., 2005;Karimi-Abdolrezaee et al., 2006), casting doubt as to whether remyelination of chronically demyelinated axons can lead to significant functional recovery. However, in neither of the chronic studies mentioned above was successful remyelination achieved following administration of cellular transplants to chronic SCI. When OPCs were injected into a contusion injury site 10 months post-injury the cells survived and differentiated into adult oligodendrocytes, but did not remyelinate demyelinated axons or result in functional improvements as they had done if administered 1 week post-injury (Keirstead et al., 2005). In the other study, transplanted NPCs were shown to differentiate into OPCs or mature oligodendrocytes, remyelinate axons and improve locomotor function if administered 2 weeks following a spinal compression injury, but the majority of injected cells failed to survive if administered 8 weeks post-injury (Karimi-Abdolrezaee et al., 2006). As remyelination was not achieved at chronic post-injury time points in these studies it remains a possibility that if successful remyelination could be achieved in chronic SCI it may be associated with functional improvements. Indeed, there is some evidence that the chronic application of myelinating cells may improve function since modest improvements in BBB scores and paw positioning were observed in

contused rats that received Schwann cell transplants 2 months following injury (Barakat et al., 2005), although anatomical evidence suggested that the beneficial effects may have been due to axon growth and/or sprouting rather than remyelination. In the present study it was found that, despite extensive remyelination at 12 weeks post-injury, there remained a small but significant proportion of demyelinated axons. Importantly, it was also demonstrated that even in chronic stages of SCI conduction can be restored to some axons upon cooling; these are most likely to be the chronically demyelinated axons observed by EM. This further demonstrates the potential of viable but non-conducting axons as important therapeutic targets. Whether such therapies should involve remyelination, or other methods of reducing conduction block, remains for further study.

Interestingly, it was also demonstrated that the myelination status of axons vary depending on their location relative to the cavity border, with more demyelinated axons found closer to the cavity border. This has not previously been studied in the rat and highlights the clinical relevance of the injury model since substantial demyelination in the pericavity region surrounded by a rim of sub-pial myelinated axons has also been observed in chronic human SCIs (Guest et al., 2005). This phenomena may arise because a number of pathological processes which contribute to necrosis of oligodendrocytes, such as vascular responses, ischaemia, excitotoxicity and inflammation (Hagg and Oudega, 2006), are likely to be maximal within and close to the cavity.

2.4.3 Schwannosis following spinal contusion

Spontaneous remyelination in the spinal cord by both Schwann cells and oligodendrocytes has been demonstrated following focal demyelinating lesions (Gilson and Blakemore, 2002) and contusion injury in rats (Totoiu and Keirstead, 2005;Keirstead et al., 2005). The presence of Schwann cells in the injured spinal cord has also been reported in humans (Bruce et al., 2000;Guest et al., 2005), particularly in chronic injuries. This is in line with the present findings, where remyelinated axons associated with Schwann cells were observed in contused spinal cords at 4 and 12 weeks after injury, but not within the first week following injury. These Schwann cells may originate in the peripheral spinal roots and migrate to the spinal cord as a result of injury-induced compromise of the central glial barrier (Franklin and Blakemore, 1993). Alternatively, they may derive from CNS-resident oligodendrocyte precursors (Zawadzka et al., 2010). Although one cannot be sure of the cellular origins of the remyelinating Schwann cells observed in this study, it is likely that they had migrated into the cord since they were only observed in the dorsal columns in close proximity to the dorsal roots; this is also the area that experienced the initial impact of the injury.

2.4.4 Behavioural changes following spinal contusion

The BBB locomotor rating scale was used in this study as it is the most widely used behavioural test for assessing functional loss following contusion injury (Basso et al., 1995;Scheff et al., 2003;Cao et al., 2005b;Nout et al., 2011). A horizontal ladder test (Metz and Whishaw, 2002) was also used to further characterise functional deficits. Interestingly, a different pattern of post-injury changes was observed using the two tests, with a differing time course of spontaneous improvements over the acute to sub-acute stages. The varying contributions of different spinal pathways, whether sensory or

motor, provides a likely explanation for the differing time course of recovery dependent on behavioural technique employed. The BBB test primarily looked at gross locomotion as the vast majority of animals remained inconsistently coordinated, meaning their score was generally limited by the level of coordination they achieved, in agreement with previous assessments of the same injury using BBB rating (Scheff et al., 2003; Springer et al., 2010). The ladder test however, required more sensorimotor integration in order for an animal to perform well (i.e. accurate foot placement, some ability to grip rung) and this is likely to have contributed to the continued improvement in this task once BBB scores had already plateaued. The time course of recovery using the ladder test more closely resembled that seen following electrophysiological assessment of sensory function. It is likely that the extended time course for recovery on the ladder is at least partially due to the continued improvement in sensory function, as aspects such as accurate paw placement and gripping the rung upon placement incorporate a sensory element. However, while both these tests provide a valuable outcome measure for assessing changes in function following spinal contusion, it should be noted that neither of these tests are purely tests of sensory function and thus don't provide a specific behavioural correlate to the electrophysiological assessment of dorsal column sensory function. Results of a purely sensory behavioural test, such as assessment of time taken for animals to notice a small piece of sticky tape stuck to the plantar surface of an affected paw, may correlate more directly with the electrophysiological findings of this study.

2.4.5 Histopathological changes following spinal contusion

Interestingly, while correlates can be drawn between behavioural function, axonal conduction and demyelination following spinal contusion, the gross histopathological

changes that occur after injury do not correlate with these parameters. Indeed, the period of endogenous repair, where spontaneous improvements in function and remyelination are observed, occurs during the period where secondary pathology (such as cavitation, cell loss and glial scarring) is progressing. Much of the pathology which follows a contusion injury is already well established (Beattie et al., 1997a; Rabchevsky et al., 2001; Baker and Hagg, 2005; Ek et al., 2010) and the current observations of progressive destruction of white and grey matter tissue and cavity formation are in line with these previous studies. The immunohistochemical examination of cell loss and glial scarring also highlighted the rapid loss of the vast majority of neuronal cell bodies in the grey matter at the lesion epicentre. Furthermore, although the phenomenon of glial scarring is well-established in the literature (Reier et al., 1983), here the transition from gross and widespread astrogliosis in acute and sub-acute stages of injury to the formation of a solid, dense glial scar surrounding the large areas of cavitation at the injury epicentre is shown, all of which contributes to an environment that is particularly hostile to regenerative growth and repair (Fitch et al., 1999; Fawcett and Asher, 1999; Fitch and Silver, 2008). It is also interesting to note that some aspects of post-injury pathology, such as cavity formation, continue to progress well into the chronic stages of injury, even after spontaneous functional improvements have plateaued.

2.4.6 Summary

These observations indicate that following spinal contusion, the immediate mass destruction of white and grey matter, cell loss and demyelination all contribute to acute conduction block. This is followed by a period of endogenous repair of axons in the spared rim in the sub-chronic phase, including remyelination and improved conduction, which correlates with improved behavioural function. Spontaneous repair reaches a

plateau however, and conduction failure, impaired behavioural function, demyelination and pathology persist into the chronic stages of injury. These studies provide valuable insight into understanding mechanisms underlying functional loss following SCI and processes of spontaneous repair and have identified an important population of viable, but non-conducting axons that remain accessible to therapies aimed at restoring conduction, even at chronic stages of a SCI.

Chapter Three

3 Chapter 3:

Using chondroitinase ABC gene therapy to promote functional repair in a thoracic contusion injury model

3.1 Introduction

CSPGs are well established as being one of the key inhibitory molecules limiting repair in the injured CNS (CSPG-mediated inhibition discussed in detail in section 1.4.3.3). CSPGs are present throughout the CNS as a major component of the ECM, as well as in the specialised condensed ECM that forms around many neuronal cell bodies known as perineuronal nets (PNNs) (Yamaguchi, 2000; Novak and Kaye, 2000). In addition to their presence in the normal adult CNS, CSPGs are also dramatically upregulated following traumatic injury to the CNS, particularly in association with the glial scar (Lemons et al., 1999; Asher et al., 2000; Asher et al., 2002; Jones et al., 2003b). This upregulation adds to the already potent inhibitory environment of the adult CNS, inhibiting regeneration of injured axons as well as sprouting of spared axons (Davies et al., 1997; Davies et al., 1999). Furthermore, any axons that manage to regenerate or sprout through the inhibitory environment are likely to be restricted in forming functional connections due to the presence of PNNs around many neuronal cell bodies. The inhibitory actions of CSPGs are primarily mediated via the interactions of their GAG side chains (reviewed in (Galtrey and Fawcett, 2007; Sharma et al., 2012). The enzymatic removal of these CS-GAGs using ChABC has been shown to alleviate many of the inhibitory effects associated with CSPGs and result in improved functional

recovery following various experimental models of SCI (Smith-Thomas et al., 1994;McKeon et al., 1995;Bradbury et al., 2002;Caggiano et al., 2005;Cafferty et al., 2008;Tom et al., 2009b;Starkey et al., 2012) (see section 1.5.7). Despite there being strong evidence showing the beneficial effects of ChABC on SCI, the majority of studies involving the use of ChABC in SCI to date have involved the use of partial section injuries (such as dorsal column crush, spinal hemisection and pyramidotomy injury models). The few studies using ChABC in more translational models, such as contusion or compression injury models, have had limited success. For example, when intraspinal injections of ChABC were administered rostral and caudal to the injury site immediately after a cervical hemicontusion injury there was no associated improvement in skilled or gross locomotor function (Tom et al., 2009a). The treatment however, did induce robust sprouting of serotonergic fibres within the grey matter both rostral and caudal to the contusion injury, suggesting that whilst a single administration of ChABC was having beneficial effects on spinal repair, these were not sufficient to result in functional improvements in a more traumatic injury model. Although no functional improvements were associated with the single ChABC administration paradigm used in this study, another study has shown that repeated intrathecal infusions of ChABC could promote functional recovery in a severe compression model of SCI induced by crushing the thoracic spinal cord with forceps (Caggiano et al., 2005). In this study improvements in both gross locomotor and bladder function were observed following repeated intrathecal ChABC infusion in particularly severe compression injuries (i.e. no stepping movements in injured animals, only individual joint movements). However, this effect was lost in compression injuries of a more moderate severity, indicating that there are limitations to this repeated infusion paradigm. ChABC has also been used as part of a combination treatment in order to try and enhance efficacy in some of these more

traumatic, translatable injury models. In one such study, ChABC was combined with a peripheral nerve graft and GDNF to promote regenerative bridging of a cervical hemicontusion (Tom et al., 2009b). Although animals receiving ChABC as part of their combination treatment achieved better recovery of locomotor function than animals receiving a combination of just GDNF and a nerve graft, the invasive nerve grafting procedure meant that all animals remained notably more impaired following therapeutic intervention than they had been when the injury had been left untreated. In another combination study ChABC was constantly delivered for 7 days via an intrathecal catheter attached to an osmotic minipump to a chronic thoracic clip compression injury site (6 weeks post-injury) and this was followed by injection of NPCs and a mixture of growth factors to the injury site (Karimi-Abdolrezaee et al., 2010). If ChABC was administered alone it led to modest collateral sprouting of the CST rostral and caudal to the injury and robust sprouting of serotonergic fibres, but this was not associated with any functional improvements. Combining ChABC with NPCs and growth factors however, further enhanced collateral sprouting of the CST and resulted in improved gross and skilled locomotor function. The limited success of ChABC in more traumatic, translational models of SCI, particularly if administered alone, highlight that there are potential issues that limit its clinical applicability. These issues derive in particular from its lack of thermostability and current sub-optimal modes of delivery.

3.1.1 Limitations of ChABC in its current form

One potential disadvantage of ChABC is that, as an enzyme, an inherent property is its restriction to a relatively fine window of conditions in which it can function optimally and remain stable. In particular, mammalian body temperature can limit the activity of ChABC and, more specifically, the length of time for which it can remain active (Tester et al., 2007). Whilst the recommended incubation conditions of 37°C and pH 8.0 for

ChABC mean that it is suited to function well at body temperature and in the conditions of the CNS, it has been shown that the CSPG degrading activity of ChABC rapidly decreases over time when incubated at body temperature (Tester et al., 2007). Results from this study indicated that although ChABC efficiently degraded CSPGs following incubation at body temperature for 24 hours, its activity was dramatically decreased after 3 days and barely detectable after 5 days. Furthermore, if the incubation temperature was slightly increased to 39°C, enzyme activity decreased even more rapidly and CSPG degradation was significantly less at 24 hours than that achieved following incubation at 37°C. This thermoinstability means that, in general, delivery of ChABC to the CNS in experimental models has been by repeated administration, via an externalised catheter or cannula (Bradbury et al., 2002;Caggiano et al., 2005;Tester and Howland, 2008) or multiple intraspinal injections (Alilain et al., 2011). Unfortunately these are unlikely to represent viable delivery methods should ChABC ever be assessed in human patients. This is primarily due to the highly invasive nature of repeated administration to the CNS and the likelihood of further exacerbating existing damage, as well as the increased susceptibility to infection caused by an externalised port to the CNS and administration of multiple doses. Ideally, ChABC would be given as a single dose and remain active for an extended period of time. Attempts to thermostabilise ChABC have met with some success, such that ChABC incubated with trehalose at 37°C successfully retained its CSPG digesting activity after incubation for up to 4 weeks (Lee et al., 2010a). Whilst this overcomes the issue of ChABC's rapid loss of function at body temperature, another potential issue remains even if using a more stable form of ChABC; although administration of thermostabilised ChABC would result in longer-term digestion of CSPGs, thereby allowing more time for axonal regeneration and sprouting in the less inhibitory environment, CSPG digestion would

still remain localised to the site of administration. Whilst this means that axonal regeneration and sprouting would be enhanced around the lesion site (if this is where the ChABC had been administered), the presence of CSPGs in the ECM throughout the CNS, albeit less concentrated than at the lesion site, means that outside of the ChABC treated area axons would still encounter CSPG-mediated inhibition. In particular, the presence of CSPGs in intact PNNs could prevent sprouting or regenerating axons from being able to form functional connections with many of their potential targets, limiting the functional benefits that could potentially have been achieved had CSPG degradation been more widespread. Thus, the effects of ChABC are likely to be optimal if its CSPG-degrading activity can be sustained over an extended period of time and its distribution is widespread, extending well beyond the area of injury. One potential mechanism through which this could be achieved is via the use of gene therapy. If the gene for ChABC could be inserted into resident CNS cells and the active protein expressed by these cells, then CSPG digestion would be sustained due to the continuous production of new ChABC, whilst secretion of ChABC from long-distance CNS axons would result in widespread distribution.

3.1.2 Viral vectors for gene therapy

The transfection of host cells with a gene of interest in order to elicit endogenous production of the protein it encodes can be successfully achieved using a variety of techniques; one of the most successful and widely used of these techniques for *in vivo* transfection is the use of viral vectors (Kay et al., 2001; Davidson and Breakefield, 2003; Abdellatif et al., 2006; Karra and Dahm, 2010; Franz et al., 2012). Viral vectors are based on natural viruses and as such can infect and hijack the cellular machinery of host cells in order to replicate their genome and assemble new viral particles. In the case of gene therapy viral vectors, the viral particles encapsulate a modified genome carrying a

therapeutic gene cassette in place of the viral genome (Kay et al., 2001). The most commonly used viral vectors for gene therapy in the CNS are herpes simplex virus (HSV), adenovirus (AdV), adeno-associated virus (AAV) and lentivirus (LV). Each vector type has its own specific properties and, accordingly, each is associated with specific advantages and disadvantages (Davidson and Breakefield, 2003; Mason et al., 2011).

HSV is a large DNA virus and as such has the largest transgene capacity of the vectors listed above (up to 50 kb) when converted into a recombinant HSV vector (Davidson and Breakefield, 2003). Whilst HSV vectors are capable of efficiently transducing a number of cell types, one of their advantages for use in the CNS is that they have a particularly high tropism for neurons. In addition there is no genome integration using these vectors, avoiding the risk of insertional mutagenesis (Dobson et al., 1990). A potential disadvantage of HSV vectors is the complexity of their attachment and entry to host cells, involving multiple viral envelope glycoproteins, making it difficult to target specific cell types (Kay et al., 2001). Additionally, low level expression of viral genes can occur using these vectors, raising the possibility of cytotoxicity or activation of the immune response (Karra and Dahm, 2010). Finally, whilst onset of expression is rapid using HSVs (within a few hours), levels of expression often decrease fairly rapidly and although mutating some of the HSV viral proteins has been shown to enhance the duration of expression (Liu et al., 2009), long-term expression is poor compared with many other vectors.

AdV is also a DNA virus and has a carrying capacity of up to ~35 kb, although AdVs with this carrying capacity require a helper virus due to the AdV lacking the genes necessary for replication (Caillaud et al., 1993; Kay et al., 2001; Karra and Dahm, 2010). One major disadvantage of AdVs is that they elicit a strong immune response,

particularly at high titres, and can be cytotoxic. Indeed, AdV toxicity was responsible for the first reported human fatality using gene therapy (Kay et al., 2001). Whilst the removal of almost the entire viral genome from AdVs, together with the use of a helper virus, has helped to reduce immune activation and cytotoxicity, concerns remain over the safety of AdVs (Tibbles et al., 2002; Karra and Dahm, 2010). Onset of expression is relatively quick using AdVs, normally occurring after 1-3 days, and there is no genome integration. Additionally, many cell types can be efficiently transduced using AdVs, although their preferential transduction of glial cells may be viewed as a potential drawback in instances where neuronal targeting is desired (Karra and Dahm, 2010).

AAV, like each of the viral vectors described above, is a DNA virus. Unlike the vectors described above however, AAVs have a very small carrying capacity of only ~5 kb and this represents the major limitation of this vector system (Goncalves, 2005). As there are no known diseases associated with AAV infection in humans, AAV vectors are generally considered a safe option for gene therapy. Whilst wild-type AAVs stably integrate into the human genome in a site-specific manner, recombinant AAV vectors rarely integrate so there is a very low risk of insertional mutagenesis (Buning et al., 2008). In addition, there are a variety of AAV serotypes, each with differing tropisms and this allows a range of cell types to be targeted using AAV vectors. This offers a distinct advantage as it allows targeting of specific cell types or of a broad range of cell types. The slow onset of expression using AAVs (often taking up to 2 weeks) could be a significant limitation in instances where acute administration of gene therapy is necessary or beneficial (Kay et al., 2001).

Lentiviral vectors (LVs) are derived from a subclass of retroviruses and have a moderate carrying capacity of ~8 kb. In contrast to other retroviruses, which can infect only dividing cells, LVs are capable of successfully transfecting non-dividing cells

(Cockrell and Kafri, 2007;Nayerossadat et al., 2012). Following *in vivo* administration, LVs display particularly low toxicity and do not illicit an immune response (Naldini et al., 1996;Kafri et al., 1997;Abdellatif et al., 2006;Karra and Dahm, 2010). Whilst LVs were originally derived from HIV-1, which has a natural tropism for lymphocytes, they can be pseudotyped. This is a process in which the genes encoding the natural envelope proteins are replaced with genes for heterologous envelope proteins from a different virus, such as vesicular stomatitis virus G protein (VSV-G) (Naldini et al., 1996;Aiken, 1997). The use of VSV-G pseudotyped LVs confers a much broader tropism, allowing a greater variety of mammalian cells to be targeted (Naldini et al., 1996). The ability of LVs to stably integrate into the host genome has both advantages and disadvantages: the integration of the genetic information in the vector into the host genome helps to achieve long-term, stable transgene expression. However, integration also carries the risk of insertional mutagenesis which can disrupt function of essential genes, potentially leading to oncogenesis in some cases (Nienhuis et al., 2006). The mutation of the viral integrase enzyme can prevent genomic integration, whilst the functions of viral assembly, nuclear translocation and reverse transcription remain unaffected. Transduction with these non-integrating LVs has been shown to lead to stable and sufficient gene expression, and is particularly effective if non-dividing cells, such as neurons, are the target for transduction (Yanez-Munoz et al., 2006;Wanisch and Yanez-Munoz, 2009;Rahim et al., 2009). In addition to the long-term transgene expression that can be achieved using LVs, onset of expression is rapid, occurring within hours of transduction (Karra and Dahm, 2010). This represents a major advantage for cases such as spinal cord injury, in which acute application of gene therapy is likely to be beneficial.

3.1.3 Gene therapy for SCI

Various methods of gene therapy have previously been assessed in animal models of SCI. Perhaps the most frequently used of these is the *ex vivo* transduction of a cellular graft, resulting in the secretion of therapeutic factors from the graft when it is transplanted at the site of injury (Franz et al., 2012). Whilst a wide variety of cell types have been utilised as genetically modified cell-based grafts, transduction of the cells to express and secrete a trophic factor has been by far the most commonly employed gene therapy strategy (Tuszynski et al., 1994;Tuszynski et al., 1997;Grill et al., 1997a;Liu et al., 2002b;Ruitenber g et al., 2003;Blesch et al., 2004;Lu et al., 2005;Cao et al., 2005a;Hollis et al., 2009b). The direct *in vivo* administration of viral vectors in order to transduce endogenous CNS cells has been a less frequently used approach to gene therapy in SCI research. When this approach has been used however, as with genetically modified cell-based grafts, trophic factors have been the most common transgene type to be administered via viral vector. For example, BDNF gene therapy following SCI has been assessed using AAVs (Blits et al., 2003;Kwon et al., 2007), AdVs (Zhou and Shine, 2003;Nakajima et al., 2010) and LVs (Kwon et al., 2007). Administration using each of these viral vectors successfully led to high levels of BDNF expression in the injured CNS and various beneficial effects were described in each study, including prevention of atrophy and apoptosis (Kwon et al., 2007;Nakajima et al., 2010), enhanced plasticity of the CST (Zhou and Shine, 2003) and improved hindlimb function (Blits et al., 2003). Importantly, the effects achieved following a single administration of a viral vector appear to, at the very least, be equal to what had previously been achieved following constant infusion of BDNF via an osmotic minipump or repeated administration via an externalised catheter (Oudega and Hagg, 1999;Bamber et al., 2001;Blits et al., 2003). However, the high levels of BDNF production associated with

administration of AAV-BDNF have also been shown to result in thermal hyperalgesia and spasticity (Boyce et al., 2012), indicating that caution must be applied when using gene therapy as a delivery mechanism. Interestingly, the spasticity resulting from AAV-BDNF treatment may well have contributed to a number of the apparent functional benefits observed, such as the ability to support body weight using the affected hindlimbs. Viral vectors have been used to mediate gene delivery of a number of other trophic factors following SCI, including NT-3 (Zhou and Shine, 2003;Zhou et al., 2003;Blits et al., 2003;Taylor et al., 2006;Fortun et al., 2009), FGF (Huang et al., 2011), GDNF (Zhou and Shine, 2003;Tannemaat et al., 2008), VEGF (Facchiano et al., 2002), NGF (Blesch et al., 2005;Tannemaat et al., 2008) and IGF-1 (Hollis et al., 2009b). Interestingly, the retrograde transport of many viral vectors makes it possible for some vectors to be administered via intramuscular injections and still result in expression of the gene of interest in the spinal cord (Fortun et al., 2009), providing a minimally invasive route of delivery. The findings of all of these studies highlight that viral vectors are an effective means of inducing high levels of expression of a gene of interest in the injured spinal cord, and that the need for only a single administration is a major benefit.

Whilst trophic factors have been the most common target for gene therapy in experimental models of CNS injury, a number of other factors have been delivered to the injured CNS using viral vectors. For example, LVs have been used to induce high levels of polysialic acid (PSA) (Zhang et al., 2007a;Zhang et al., 2007b). PSA is a large molecule important in neural cell adhesion, and these studies have shown that enhancing its expression using an LV leads to enhanced penetration of regenerating sensory axons across the lesion border. Expression levels of receptor proteins can also be targeted using viral vectors. For example, the delivery of *trk-B* via LV was shown to enhance regeneration of CST axons following a sub-cortical lesion, due to activation of

ERK signalling (Hollis et al., 2009a). Attempts have also been made to deliver ChABC to the injured spinal cord using viral vectors. Due to its prokaryotic origins however, ChABC secretion is significantly impaired in mammalian systems due to inappropriate processing by the mammalian translation system; this results in the ChABC protein being heavily glycosylated and prevents secretion of functional enzyme (Muir et al., 2010). Attempts have therefore been made to deliver a different form of chondroitinase, known as chondroitinase AC (ChAC), to the mammalian spinal cord using a LV (Jin et al., 2011). ChAC however, is less effective than ChABC at cleaving CS-GAG side-chains from CSPGs and the specific activity of the secreted product using this LV is notably low (Curinga et al., 2007). A recent study carried out site-directed mutagenesis of the bacterial ChABC gene to prevent the heavy glycosylation normally observed following mammalian translation (Muir et al., 2010). The modified ChABC gene was successfully translated and secreted by mammalian cells and, most importantly, the secreted ChABC showed high levels of activity. The successful modification of the bacterial ChABC gene to allow secretion of active enzyme from mammalian cells has opened the door to the possibility of ChABC gene delivery in experimental models of SCI.

Direct application of a viral vector containing the modified ChABC gene represents an ideal mechanism of ChABC gene delivery as this would allow widespread expression of ChABC rather than just at the injury site, as would be the case if genetically modified cells were transplanted. Additionally, intraparenchymal injection of viral vectors has been shown to lead to higher levels of gene expression than if the vector is administered intrathecally (Mannes et al., 1998). In terms of which viral vector would be optimal for ChABC gene delivery, the widespread, long-term and stable transgene expression that can be achieved using either LVs or AAVs makes these

vectors the prime candidates. As ChABC has been shown to be more effective if given at an early post-injury time-point, the rapid onset of expression following transduction using an LV gives these vectors a distinct advantage over the comparably slow expressing AAVs in this particular situation. In this chapter I will, therefore, assess the efficacy of ChABC gene therapy using a LV in a clinically relevant model of SCI. Animals will receive a thoracic contusion injury, as characterised in the previous chapter, followed by acute administration of a LV containing either the modified ChABC transgene or a control GFP transgene. Post-injury functional and anatomical changes will then be assessed using the techniques described and developed in the previous chapter. Additionally, the effects of ChABC gene therapy on plasticity will be assessed using histological and electrophysiological techniques. The primary findings of this chapter are that long-term and widespread CSPG digestion can be achieved using LV-mediated ChABC gene therapy and that this leads to significant improvements in spinal conduction, behavioural function, injury pathology and spinal plasticity. Additionally, ChABC gene therapy is linked to modulation of the immune response, providing a potential underlying mechanism for some of the observed improvements.

3.2 Materials and methods

3.2.1 Chondroitinase gene

The *Proteus vulgaris* chondroitinase ABC (ChABC) gene was modified (by E.M.Muir, University of Cambridge) with mutations targeted to remove five cryptic N-glycosylation sites and addition of a mammalian signal sequence (Muir et al., 2010), and resynthesised with mammalian preferred codons.

3.2.2 Lentiviral vectors

The modified cDNA was subcloned into three lentiviral transfer vectors named as LV-1, LV-2 (both with cytomegalovirus immediate-early [CMV] promoter; produced by J.Verhaagen, Netherlands Institute of Neuroscience and R.J.Yáñez-Muñoz, Royal Holloway (University of London), respectively), and LV-3 (with the mouse phosphoglycerate kinase [PGK] promoter; produced by B.L.Schneider, École Polytechnique Fédérale de Lausanne). The production of these vectors is described in detail elsewhere (Zhao et al., 2011). The resulting vectors were integrating, self-inactivating vectors, pseudotyped with VSV-G. Viral particles were concentrated by ultracentrifugation and titered by a p24 antigen ELISA assay (LV-1 and LV-3) or Q-PCR (LV-2). The viral titer of LV-1 was 99 µg/ml of P24, corresponding to $\sim 6 \times 10^5$ TU/µg; the viral titer for LV-2 was 1.44×10^9 gc/ml, corresponding to $\sim 8 \times 10^5$ TU/µl; the viral titer of LV-3 was 479 µg/ml of P24, corresponding to $\sim 10^6$ TU/µl. Control virus was generated from the same transfer vectors containing the cDNA coding for GFP.

3.2.3 Intraspinal injections

All procedures were performed in accordance with the UK Animals (Surgical Procedures) Act 1996. Anaesthetized (ketamine, 60 mg/kg, and medetomidine, 0.25

mg/kg) adult female Sprague Dawley rats received single (1 μ l) intraspinal injections of either protease free ChABC enzyme (Seikagaku Corporation; 10U/ml, n=4), LV-ChABC (LV-1, n=6; LV-2, n=6; LV-3, n=13) or saline (n=8). At 2 and 8 weeks post-injection n=3 from each group, except saline, were perfused in order to assess CS-GAG digestion. n=8 and n=7 from the saline and LV-3 group, respectively, were assessed behaviourally for pain sensitivity over the course of 12 weeks. For LV-3 only, CS-GAG digestion was also assessed at additional time points of 6 hours, 24 hours, 6 months and 1 year post-injection (n=2 per time point).

3.2.4 Spinal cord injury and treatment

Anaesthetized (ketamine, 60 mg/kg, and medetomidine, 0.25 mg/kg) adult female Sprague Dawley rats received a midline 150 kdyne spinal contusion injury at spinal level T10/11 using an Infinite Horizon's impactor (Precision Systems Instrumentation). Immediately after contusion injury rats received intraspinal injections (0.5 μ l at 1 mm rostral and caudal of the injury site) of either ChABC (10 U/ml), LV-ChABC (LV-1, LV-2, or LV-3), or LV-GFP, whilst an additional control group received no injection (contusion only). One cohort of animals were used for terminal electrophysiology and histology at 2 weeks post-injury (n=4 all groups), whilst another cohort were used for electrophysiology and histology at 10 weeks post-injury (n=4; LV-1, LV-2 and ChABC, n=15; contusion only, LV-GFP, LV-3). Behavioural analyses were performed over 10 weeks post-injury in part of this cohort (n=15; contusion only, LV-GFP, LV-3). Following the final behavioural time point, n=5 from each of these three groups received a 4 μ l injection of 1% cholera toxin β subunit (CTB) into the left sciatic nerve to unilaterally label large, myelinated A β fibres in the dorsal columns of the spinal cord (the fibres being assessed using the electrophysiology protocol described in 3.2.5.1). Unfortunately there was a high level of variability in relation to the successful labelling

of appropriate fibres using this technique, fibre tract tracing data is therefore not presented. In addition to the animals that were used for functional and histological assessments, a further group of animals were used at 3 days post-injury (n=4; ChABC and LV-3) and 2 weeks post-injury (n=4; ChABC and LV-3) for biochemical analyses. All functional and anatomical analyses were carried out blinded to treatment.

3.2.5 Electrophysiology

3.2.5.1 Conduction of sensory fibres through the injury site

Axonal conduction was assessed by antidromic stimulation of sensory fibres in the dorsal columns in terminal electrophysiology experiments. The spinal cord was exposed from T7 to L5 (vertebral levels) in urethane-anaesthetized rats (1.25 g/kg). Antidromic unitary activity was recorded and quantified from teased dorsal root filaments (left and right L3-S1 roots), whilst first stimulating the entire dorsal columns 5 mm below and then 5 mm above the injury site, as described in chapter two. Conduction through the injury site was measured by calculating the number of single units present when stimulating above the injury site as a percentage of the total number of single units when stimulating below. Stimulation was continuous during recording sessions and was carried out using 0.2 ms duration, square wave pulses at a frequency of 1 Hz and an incrementally increasing intensity (0-800 μ A). Stimulation was maximal by 400-500 μ A (i.e. no further unitary activity could be recruited), but continued to be increased to 800 μ A to ensure no high-threshold fibres were undetected.

3.2.5.2 Plasticity of hindlimb spinal reflexes

Rats were terminally anaesthetised, as above, and the left sciatic nerve and its branches exposed. The motor nerve to biceps femoris (BF) and the sural nerve (SN) were dissected free and cut distally. The common peroneal nerve (CP) was dissected free but

left intact. Whole nerve recordings were individually made from the BF and the CP whilst electrical stimulation was applied to the SN. A train of 25 stimuli was delivered to the SN at a stimulus intensity of 50 μ A, 100 μ A or 300 μ A (A-fibre), or 4 mA (C-fibre) (1ms square wave pulse at a frequency of 0.5Hz). Recordings of BF or CP activity for 1 second after each impulse were captured using LabChart software (AD Instruments). Nerve activity during the 20 seconds preceding and the 50 seconds following each wind up trial was also recorded. At each stimulation intensity 3 trials were carried out on each nerve, with an interval of 5 minutes between trials to allow the nerves to return to baseline levels of resting activity. Trials always began with the lowest (50 μ A) and finished with the highest stimulation intensity (4 mA). For the BF, multi-unit recording of all spikes 25% greater than the mean noise level was made, typically producing ~5 spikes per second under resting conditions. For each animal, recording parameters were not changed between BF and CP recordings and it was noted that the CP had higher resting levels of activity, typically ~100 spikes per second. To quantify wind-up from spike frequency plots, area under the curve analysis was performed. This value was then normalised to baseline nerve activity levels and input (the number of spikes discharged during the second following the first stimulus). The normalisation serves to control for differences in recording conditions and nerve excitability and is calculated as follows:

$$\text{Wind-up} = (\text{total spikes}) - ((\text{input} \times 25) - (\text{basal} \times 25))$$

Total spikes = sum of spikes recorded during the stimulating period. Basal = average number of spikes per second in the 20 seconds prior to the stimulating period.

3.2.6 Behavioural assessments

Hindlimb function and sensorimotor integration were assessed using the horizontal ladder test, which involved counting the total number of hindlimb footslips over the course of three runs across a one metre ladder with unevenly spaced rungs, as described

in chapter two. Open field locomotor scores were also carried out according to the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Mechanical thresholds of the left forepaw (uninjured animals with cervical injections) or hindpaw (T10 contused animals) were determined by measuring latency to withdrawal using an automated electronic Von Frey, where an increasing force up to 50 g over a 20 s time ramp was applied. Thermal thresholds were determined by measuring latency to nocifensive paw withdrawal from a radiant heat source. Response to a noxious stimulus was assessed by injecting 50 µl of 5% formalin (37% in sterile saline) subcutaneously into the left forepaw footpad and quantifying the time spent licking or biting the affected paw over 45 minutes.

3.2.7 Tissue processing and immunohistochemistry

Perfusion, tissue processing and immunohistochemistry were carried out as described in chapter two. Sagittal (20µm) and transverse (20µm) frozen spinal cord sections were immunostained using the following primary antibodies: rabbit polyclonal anti-GFAP to label reactive astrocytes (1:2000, DakoCytomation), mouse monoclonal anti-NeuN to label neuronal cell bodies (1:500, Millipore), mouse monoclonal anti-NF200 to label axons (Clone N52, 1:400, Sigma-Aldrich), mouse monoclonal anti-RECA-1 to label endothelial cells (1:200, Abcam), goat polyclonal anti-CD206 to identify M2-type macrophages (Kigerl et al., 2009) (1:100, R&D Systems), mouse anti-CD68 to identify phagocytic inflammatory cells (1:100, Abcam), rabbit polyclonal anti-5-HT (1:15000, ImmunoStar), and mouse monoclonal anti-chondroitin-4-sulfate (C-4-S) to reveal digested sugar stub regions (1:5000, MP Biomedicals). For anti-C-4-S staining tyramide signal amplification was employed. Complementary secondary antibodies were donkey anti-mouse Alexa 488 (1:1000, Invitrogen), donkey anti-rabbit Alexa 546 (1:1000, Invitrogen) and donkey anti-goat Alexa 488 (1:1000, Invitrogen). After washing in PBS

slides were then coverslipped with Vectashield fluorescent mounting medium (Vector laboratories) and images were taken on a Zeiss LSM 710 upright confocal microscope at the same settings and in single sessions.

3.2.8 Anatomical quantifications

Cavity size was quantified for n=4 per group using the protocol described in chapter 2. Using Axiovision software quantification of NeuN+ cells was carried out on 10 sections per animal, spanning 2 mm through the lesion epicentre (200 μ m between sections) and giving a total value of NeuN+ cells for all 10 sections combined (n=4 per group). Quantification of 5-HT and chondroitin-4-sulfate (C-4-S) intensities was carried out in transverse lumbar spinal cord sections (n=4 per group, n=3 sections per animal at L5). All sections were photographed under the same exposure. For 5-HT the mean pixel value inside a marked area spanning around the ventral horn was measured for each section. For C-4-S three areas were marked and measured in each section, giving a mean pixel value for each section. This analysis was carried out using NIH Image J (version 1.38; National Institute of Health, Bethesda, MD, USA).

3.2.9 Electrophoresis and Immunoblotting:

For immunoblotting, rats were euthanized with a lethal injection of sodium pentobarbital and perfused with 150ml of PBS supplemented with 12.5mM EDTA to inhibit platelet aggregation and metalloproteinase activity. 5-6mm spinal cord segments were carefully dissected at T10 level to include the entire injury epicenter, weighed and placed on ice-cold PBS, supplemented with 12.5 mM EDTA and a broad proteinase inhibitor cocktail (P8340, Sigma). Prior to protein extraction spinal cord specimens were washed 3 times with the PBS solution to minimize contamination with plasma proteins. To extract cellular proteins samples were first incubated in a buffer containing 0.08% SDS, 12.5mM EDTA and proteinase inhibitors, for 4h at room temperature.

Then, matrix proteins were solubilised in 4M guanidine supplemented with 12.5mM EDTA and proteinase inhibitors (24h at room temperature). Proteins were precipitated in 100% ethanol for 16h, to remove guanidine. Cellular and ECM protein extracts were denatured and reduced in 4x sample buffer containing 500mM Tris, pH 6.8, 40% glycerol, 0.2% SDS, 2% β -mercaptoethanol, and 0.02% bromophenol blue and boiled at 98 °C for 10 min. 25 μ g of protein per sample were loaded and separated on 15-well, Bis-Tris 10% polyacrylamide gels (NuPAGE, Invitrogen). Proteins were then transferred on nitrocellulose membranes. Membranes were stained with Ponceau Red stain to visualize successful transfer and protein loading, blocked in 5% fat-free milk powder in PBS and probed for 16 h at 4 °C with a mouse primary antibody against C-4-S GAG stubs (MP Biomedicals). The antibody was used at 1:500 dilution in 5% BSA PBS. Following 3 washes in PBS tween 20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Dako Cytomation) in 5% milk powder at 1:2000 dilution for 1 h at room temperature. Finally, blots were treated with enhanced chemiluminescence reagents (ECL, GE Healthcare) and films were developed in a Kodak processor. Densitometry of developed blots was performed using ImageJ and values were normalized to beta-actin.

3.2.10 Statistical Analysis

All numerical values are reported as mean \pm s.e.m. and all statistical analyses were carried out using GraphPad Prism v5 software. Behavioural data were analyzed by two-way RM ANOVA whilst histological, immunoblot and electrophysiological data were analyzed by one-way ANOVA in instances of multiple comparisons and by unpaired Student's t-test in instances of single comparisons. Post-hoc comparisons were carried out where appropriate; all statistical tests are stated in the text.

3.2.11 Additional contributions

Immunoblotting was performed by Dr Athanasios Didangelos. Immunohistochemistry for the images in figures 3.1-3.4+3.13 was performed by Dr Katalin Bartus.

3.3 Results

3.3.1 Comparing lentiviral vectors driven by a CMV or a PGK promoter

To assess efficacy of gene delivery after spinal contusion a comparison was made between LVs driven by either CMV or PGK promoters; these either expressed GFP or engineered ChABC (e-ChABC). To determine transduction efficiency, GFP immunoreactivity was assessed in sagittal sections spanning the lesion epicentre 8 weeks after spinal contusion followed by immediate injection of LV-CMV-GFP or LV-PGK-GFP. The PGK promoter led to efficient transduction of spinal neurons, with numerous GFP-labelled neurons apparent in the spinal grey matter for several millimetres rostral and caudal to the injury site and many GFP-labelled axons in white matter tracts (Fig. 3.1A-C). GFP-labelled axonal projections were visualised as far caudal as the lumbosacral cord, where profuse labelling of spinal axons and arborizing terminals were apparent (Fig. 3.1D). GFP expression under the CMV promoter showed a different pattern, with GFP-labelling observed predominantly in glial cells around the injury epicentre and little evidence of neuronal transduction (Fig. 3.1E-G) and no evidence of transport in caudal segments of the spinal cord (Fig. 3.1H). The efficacy of CMV- or PGK-driven LVs in delivering mammalian-compatible e-ChABC to the uninjured adult rat spinal cord was next compared. It was found that a single intraspinal injection of LV-ChABC led to sustained digestion of CS-GAGs, evident by intense C-4-S stub immunoreactivity, indicating the presence of degraded CSPGs at 2 and 8 weeks following injection. In accordance with the pattern of transduction observed with the GFP gene, the LV-ChABC driven by the PGK promoter led to the most widespread areas of digested CSPGs, in comparison to the lower levels observed with CMV-driven LVs and the limited activity of ChABC enzyme (Fig. 3.2A).

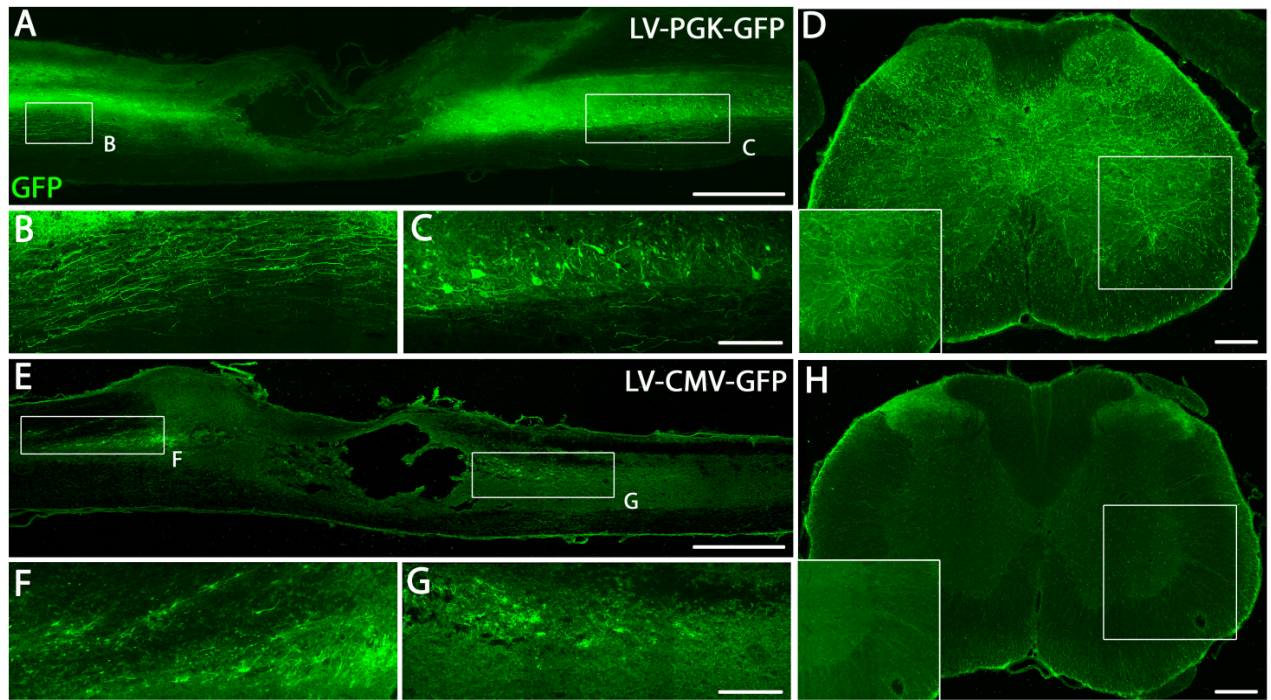


Figure 3.1. Pattern of GFP expression using LVs under a PGK or CMV promoter. GFP immunohistochemistry in sagittal sections through the contusion injury site and transverse sections of lumbar cord 8 weeks following intraspinal injection of GFP-expressing LVs under a PGK (**A-D**) or CMV (**E-H**) promoter. (**A**), The PGK promoter leads to efficient transduction of spinal neurons and axonal projections, evident by abundant GFP immunoreactivity (green) in neuronal cell bodies and axons. High magnification images of the boxed areas in (**A**) show examples of GFP-labelled spinal axons (**B**) and neurons (**C**). (**D**) GFP-labelling is observed in axonal projections as far caudal as the lumbar spinal cord following thoracic injection (insert shows high magnification of GFP-labelled axons). (**E**) The CMV promoter leads to transduction predominantly in glial cells close to the injury and injection site (GFP-labelled glial cells in boxed areas shown in high magnification in **F,G**) and no GFP-labelling is observed in the lumbar spinal cord following thoracic injection (**H**, insert shows high magnification of boxed area). Scale bars, 1 mm (**A+E**); 500 μ m (**B,C,F+G**); 250 μ m (**D+H**).

Furthermore, enhanced CSPG digestion appeared to correlate with improved spinal conduction in this preliminary assessment of delivery systems (Fig. 3.2B). Superior CSPG digestion with LV-ChABC compared to standard bacterial enzyme was confirmed quantitatively, with Western blot analysis of tissue homogenates revealing abundant C-4-S stubs at the injury epicentre and in distal areas of the spinal cord (but not brain) following contusion and LV-ChABC injection, compared to standard bacterial enzyme injection where significantly lower levels of digested C-4-S stubs were achieved and treatment was restricted to the epicentre only (Fig. 3.3A+B). Interestingly, abundant CSPG digestion within the injury epicentre at 2 weeks was associated with evidence of tissue preservation at 8 weeks following LV-ChABC injection (Fig. 3.3C+D). Conversely, following bacterial ChABC injection low levels of digested CSPGs were observed within the epicentre at 2 weeks and by 8 weeks post-injury the epicentre had developed into a large cavity devoid of any astrocytic processes (Fig. 3.3C+D), providing initial evidence that large-scale CSPG digestion achieved by LV-ChABC may reduce the development of cystic cavities following SCI. Since the LV-ChABC driven by the PGK promoter was the most potent for achieving delivery of e-ChABC to the adult rat spinal cord, this vector was used in all subsequent experiments, with LV-PGK-GFP as a control. To assess long-term expression of mammalian-compatible e-ChABC, animals were followed up to one year after a single intraspinal injection (1 μ l) of LV-ChABC (Fig. 3.4). C-4-S immunoreactivity was examined in serial sagittal sections spanning the injection site at various post-injection time points (6h, 24h, 2wk, 8wk, 6 months and one year). At 6 hours post-injection there was no evidence of C-4-S immunoreactivity. C-4-S immunoreactivity was first apparent at 24 hours, became widespread by 2 weeks and was maintained thereafter at high levels, with C-4-S still apparent even at one year post-injection (Fig. 3.4). These data indicate

stable and long-term expression of the transgene and secretion of active ChABC for at least one year following intraspinal injection of LV-ChABC.

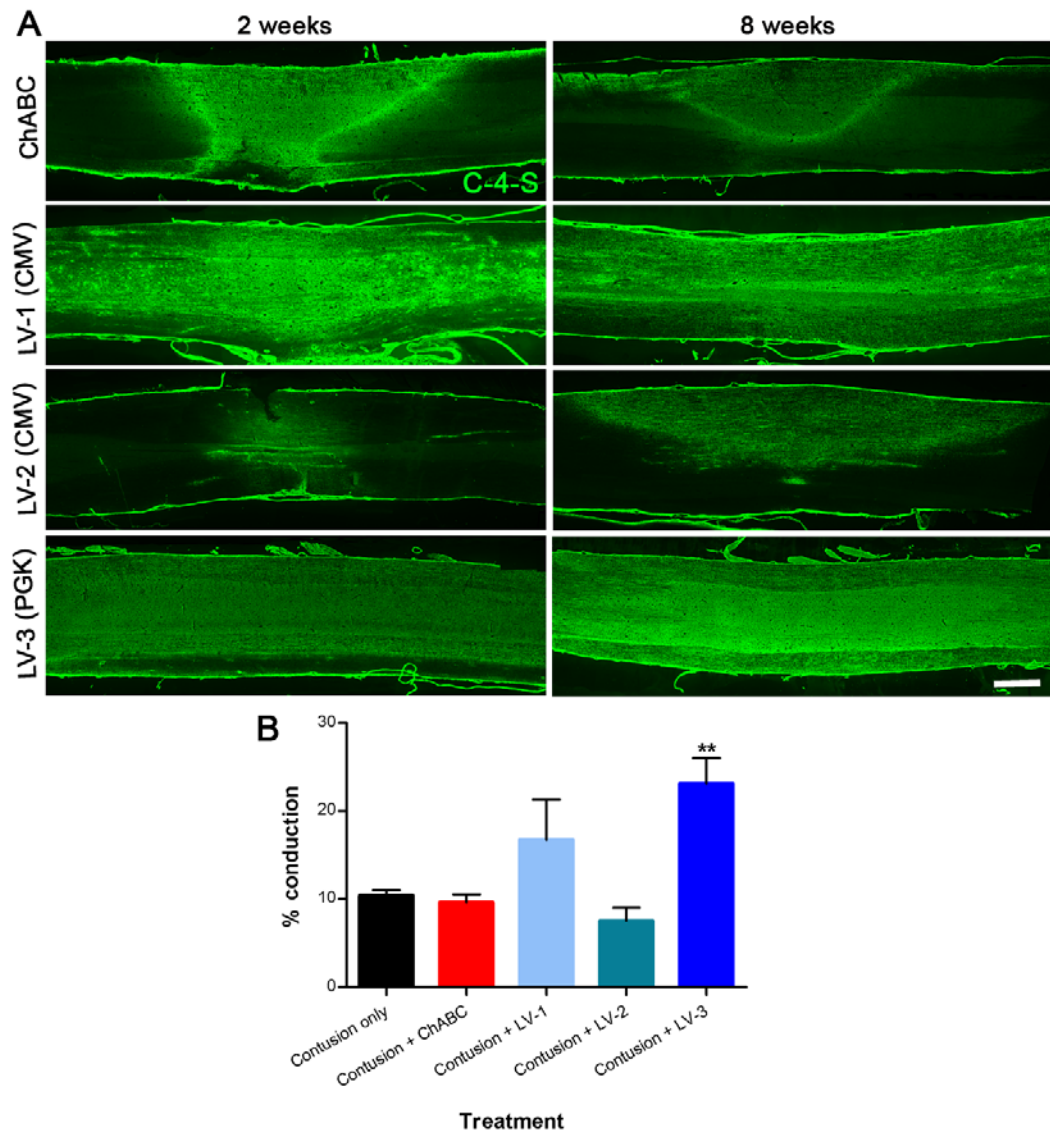


Figure 3.2. Large-scale CSPG digestion achieved with lentiviral vector delivery corresponds with increased spinal conduction following contusion injury. (A), C-4-S immunoreactivity (green) in sagittal sections 2 and 8 weeks following a single intraspinal injection of bacterial ChABC enzyme or e-ChABC packaged into either CMV- or PGK-driven LVs. Following injection of bacterial ChABC, C-4-S immunoreactivity remained localised to the injection site and activity diminished over time. In contrast, LV delivery of ChABC led to widespread areas of digested CSPGs and long-term activity, indicated by intense C-4-S

sustained at 8 weeks following injection. Note the different CSPG digestion patterns among the three LVs, with LV-3 (under a PGK promoter) eliciting the most widespread CSPG digestion. **(B)** Assessment of spinal conduction 8 weeks following a T10 spinal contusion and intraspinal injection reveals that the degree of CSPG digestion corresponds to changes in spinal conduction, with a significant increase in the percentage of axons that could conduct across a contusion injury observed in animals injected with LV-3 ($24.7 \pm 4.3\%$ compared with $10.2 \pm 0.8\%$ following contusion only). No improvements in spinal conduction were observed following contusion and delivery of bacterial ChABC enzyme, or LV-2 ($9.6 \pm 0.9\%$ and $7.6 \pm 1.5\%$, respectively) and while there was a trend for increased conduction in LV-1 injected animals ($16.7 \pm 4.5\%$), this did not reach significance (although was also not significantly different from the LV-3 treated group). Thus, the extent to which conduction was restored corresponded closely with the CSPG digestion pattern, with increased spinal conduction only observed in animals with large-scale CSPG digestion. (n=5 per group; asterisk indicates a significant difference compared to contusion only group; $p < 0.01$; one-way ANOVA, Tukey's *post-hoc*). For all figures error bars represent SEM. Scale bar, 1 mm.

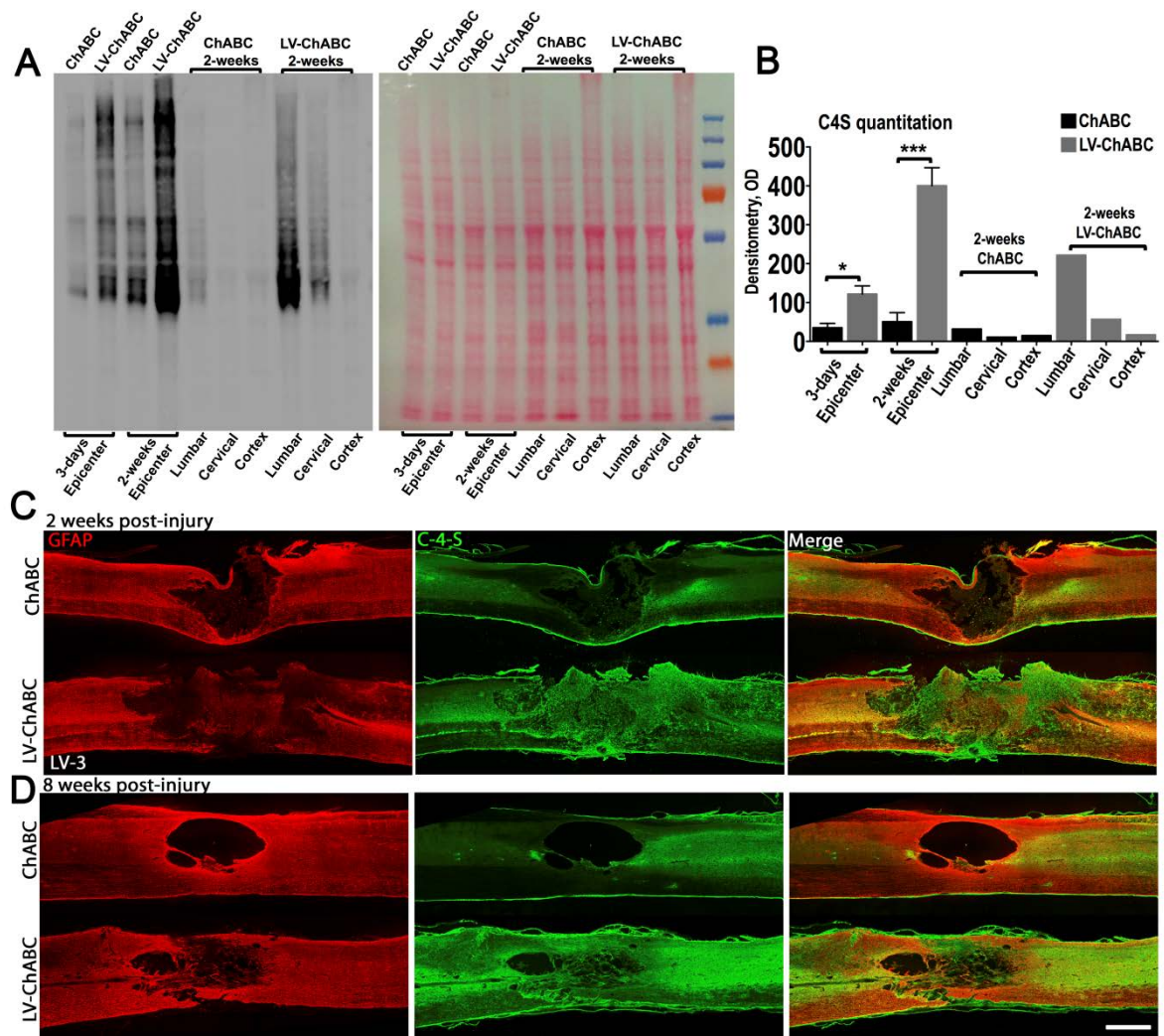


Figure 3.3. Comparison of bacterial ChABC versus LV-ChABC. (A), Immunoblotting for C-4-S stubs was used to compare GAG digestion in the injury epicentre (T10) of conventional ChABC enzyme and LV-ChABC treated cords 3 days and 2 weeks after spinal contusion. Immunoblotting was also performed on lumbar (L2) and cervical (C4) segments, as well as brain cortex, to visualize the spread of GAG digestion at 2 weeks post injury. Prior to immunoblotting, membranes were stained with Ponceau red (A, right panel) to demonstrate equal protein loading. Each lane is a pool of extracts derived from 3 animals. (B) Quantification of C4S immunoreactivity was compared by densitometry. * $p < 0.05$, *** $p < 0.001$; one-way ANOVA and Bonferroni's *post-hoc*. (C+D) Immunohistochemistry in sagittal sections through the injured thoracic spinal cord showing the C-4-S digestion pattern (green) and co-localisation with the astrocytic marker GFAP (red) at 2 weeks (C) and 8 weeks (D) post-injury. Abundant

CSPG digestion was observed at the injury epicentre 2 weeks following contusion and LV-ChABC injection. This area, which is normally devoid of astrocytes, contained many astrocytic processes, which were still present at 8 weeks following contusion. In comparison, the injury epicentre following intraspinal injection of conventional ChABC enzyme contained little evidence of digested CSPGs at the sub-acute (2 week) time point and by 8 weeks post-injury the epicentre had developed into a large cavity devoid of astrocytic processes. This data suggests that large-scale CSPG digestion achieved by LV-ChABC may have a neuroprotective effect. Scale bar, 1mm.

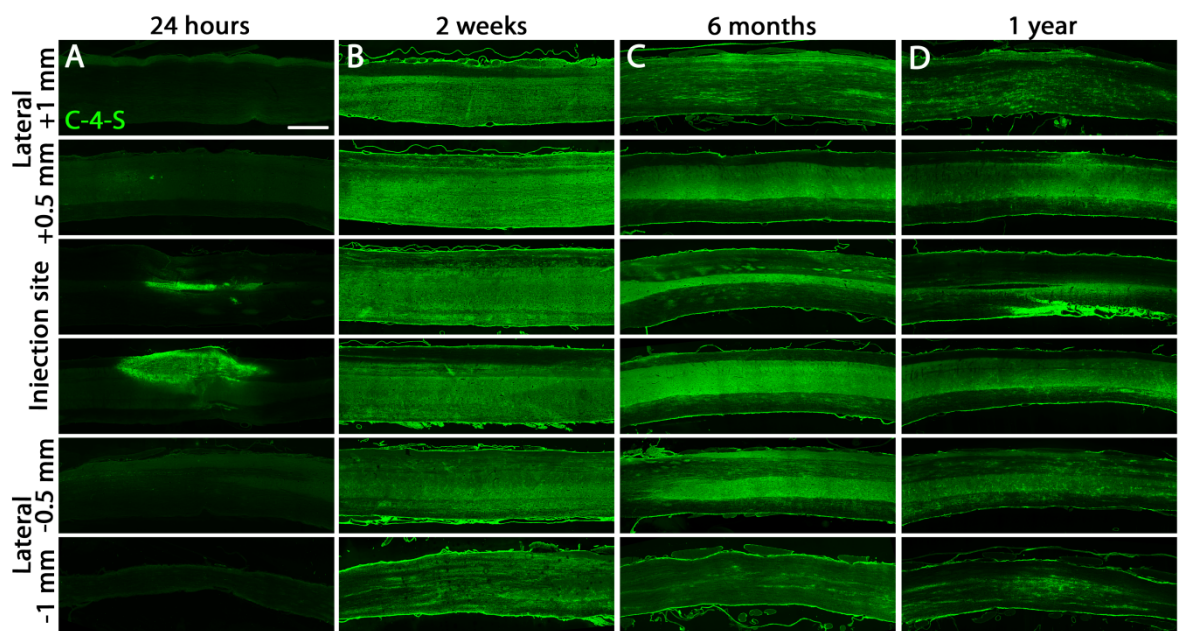


Figure 3.4. Time course of CSPG digestion by LV-ChABC under a PGK promoter in the uninjured thoracic spinal cord. (A-D) C-4-S immunoreactivity (present only where CSPGs have been degraded, green) is first detected 24 hours following intraspinal injection (A), where it is localised around the injection site, and becomes more widespread over time, with intense C-4-S immunoreactivity apparent throughout the lateral axis of the spinal cord 2 weeks following injection (B) and persisting at 6 months (C) and 1 year (D) post-injection, indicating stable long-term delivery of active ChABC. Scale bar, 1 mm.

3.3.2 The effects of LV-ChABC on lesion pathology

Potential neuroprotective effects of large-scale CSPG digestion were investigated in more detail by examining whether LV-ChABC could reduce the progressive pathology that occurs following spinal contusion, and that is largely responsible for the permanent loss of function (Schwab and Bartholdi, 1996). Stereological quantification of eriochrome cyanine staining in serial sections from 3.6 mm rostral to 3.6 mm caudal to the injury epicentre at a chronic (12 weeks) post-injury time point revealed a remarkable reduction in cavity size apparent at the lesion epicentre following LV-ChABC treatment (1 μ l injected at the time of injury), compared to untreated or LV-GFP treated animals (Fig. 3.5A-C) and significantly reduced spread of cavitation ($p < 0.01$ two-way RM ANOVA; Fig. 3.5D and Fig. 3.6). Since contusion injury leads to mass destruction of spinal grey matter, extensive neuronal cell loss typically occurs along the rostrocaudal extent of the injury, with the lesion epicentre characteristically devoid of spinal neurons. While this pattern was observed in untreated (Fig. 3.5E,A'+B') and LV-GFP treated animals 12 weeks following spinal contusion, many neuronal cell bodies were apparent at the injury epicentre and in rostrocaudal spinal segments following LV-ChABC treatment, corresponding to areas of reduced pathology and cavitation (Fig. 3.5F,C'+D'), revealed by GFAP and NeuN immunohistochemistry in serial sections through the injury. Quantification of the number of surviving spinal neurons (total cell counts from 10 serial sections from 1mm rostral to 1mm caudal to the epicentre) revealed a significant ~50% increase in the number of spinal neurons surviving the trauma following LV-ChABC treatment, compared to contusion only and contusion plus LV-GFP (3084 ± 273 , 1750 ± 359 and 2014 ± 353 NeuN positive cells, respectively; $p < 0.05$; one-way ANOVA, Tukey's *post-hoc*; Fig. 3.5I). LV-ChABC also led to

prominent changes in reactive gliosis at the lesion epicentre, with GFAP immunoreactive glial processes more diffuse and elongated following LV-ChABC, compared to the thick border of reactive astrocytes typically observed around the cavity borders following contusion only or treatment with control LV-GFP (Fig. 3.5E+F). To ascertain whether tissue at the epicentre was viable and contained axonal projections, vasculature with RECA-1 (an endothelial cell marker) and spinal axons with NF 200 (an axonal marker) immunohistochemistry were examined in longitudinal sections through the injury site. Confocal images of contused spinal cords at the lesion epicentre revealed that the much reduced lesion epicentre was well vascularised following LV-ChABC treatment, with numerous blood vessels apparent through the lesion core (Fig. 3.5G+H). Furthermore, NF 200-positive axons were abundant in the lesion epicentre following LV-ChABC treatment, in stark contrast to the axon-free large cavities in untreated and LV-GFP treated contused spinal cords (Fig. 3.5J-L). Thus, a single administration of LV-ChABC at the time of injury prevented much of the hallmark secondary pathology that is typically observed in chronic spinal cord injured tissue.

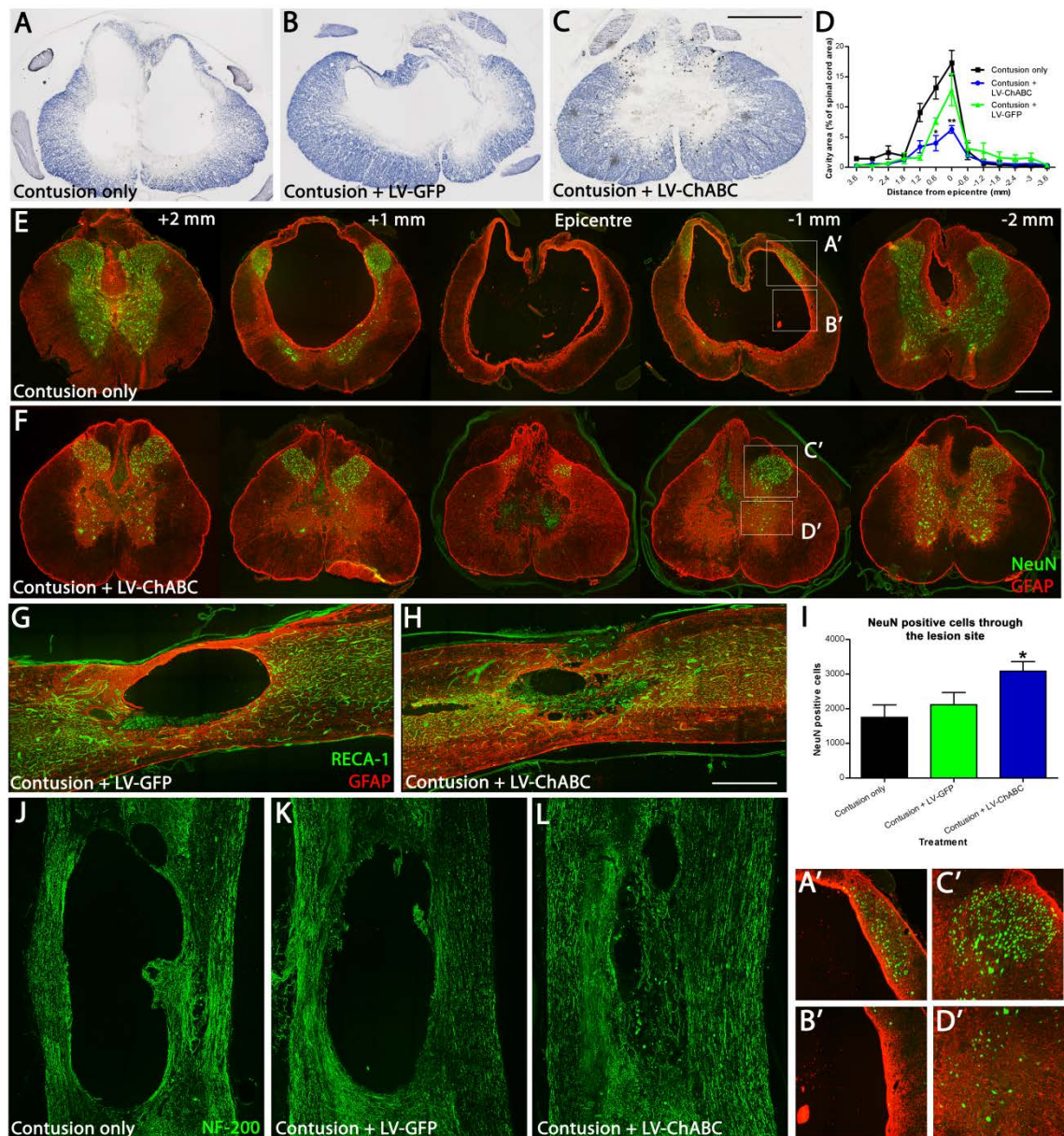


Figure 3.5. Gene delivery of ChABC leads to improved injury pathology and neuroprotection following spinal contusion. (A-C), Eriochrome cyanine histochemistry in transverse spinal cord sections 12 weeks following spinal contusion shows a marked reduction in cavity formation at the lesion epicentre following LV-ChABC treatment (C) in comparison to contusion only (A) or LV-GFP treatment (B). (D) Quantification of cavity area reveals a significant reduction in cavity size at the epicentre following LV-ChABC treatment as well as in the rostrocaudal spread of the cavity (asterisks indicate a significant difference compared to both contusion only and contusion plus LV-GFP; $p < 0.05$, two-way RM ANOVA, Bonferroni

post-hoc). There is a small, but significant, effect of the control LV-GFP treatment on reducing the cavity size at 0.6 and 1.2 mm rostral to the epicentre, although the cavity size at the epicentre was not significantly reduced compared to untreated contused animals. **(E+F)** Merged images of GFAP (astrocytes, red) and NeuN (neurons, green) immunoreactivity throughout the rostrocaudal axis. Dramatic changes in reactive gliosis and neuronal cell survival after contusion injury and LV-ChABC treatment **(F)** compared to contusion only controls **(E)** were observed. **(I)** Quantification of total NeuN-positive cells counted over a series of sections from 1 mm rostral to 1 mm caudal to the epicentre revealed a significant preservation of spinal neurons following LV-ChABC treatment, compared to contusion only and LV-GFP treatment; $p < 0.05$, one-way ANOVA, Tukey's post-hoc. **(G+H)** The much reduced lesion epicentre in LV-ChABC treated spinal cord contains numerous blood vessels, indicated by RECA-1 immunoreactivity (green). **(J-L)** While large cystic cavities are apparent in chronically injured spinal cords (12 weeks) that are untreated or received LV-GFP treatment, numerous NF 200-positive axons (green) are apparent coursing through the lesion epicentre in LV-ChABC treated spinal cords. **(A'-D')** High magnification images of the areas indicated in **(E)** and **(F)**. Scale bars, 500 μm .

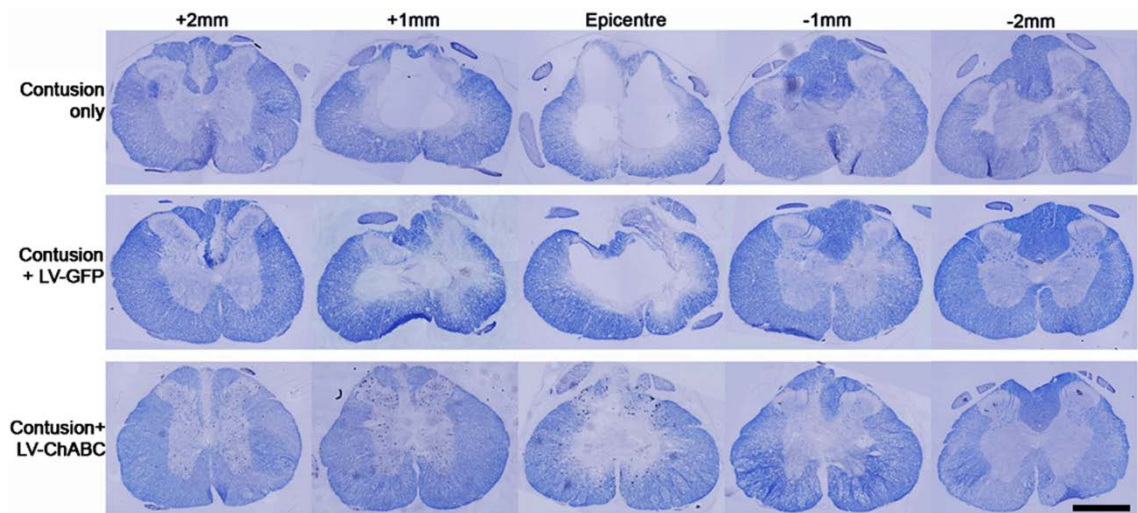


Figure 3.6. LV-ChABC reduces cavity formation following spinal contusion. Eriochrome cyanine staining of serial sections from 2 mm rostral to 2 mm caudal to the lesion epicentre illustrate the reduction in cavity formation and spread of cavitation following treatment with LV-ChABC at a chronic (12 week) post-injury time point, compared to animals with a contusion only or contusion plus LV-GFP treatment. Scale bar, 500 μ m.

3.3.3 The effects of LV-ChABC on behavioural function and spinal conduction

It was also examined whether LV-ChABC would improve functional outcome following spinal contusion. Force readouts from individual animals confirmed consistent reproducible injuries across all experimental groups (Fig. 3.7A) and BBB locomotor rating confirmed that animals in all groups were equally impaired immediately following contusion injury, with BBB scores not significantly different between groups at two days post-injury (Fig. 3.7B). However, BBB scores did not reveal any improved locomotor function following LV-ChABC treatment, with all animals reaching a plateau at a score of 13 and no significant group differences over time ($p > 0.05$ two-way RM ANOVA; Fig. 3.7B). This lack of recovery was unexpected, given the evidence of tissue preservation and neuroprotection following LV-ChABC treatment and suggests there is a limit to the extent of recovery with this treatment. However, functional effects were also assessed using the horizontal ladder test, a skilled motor task requiring sensorimotor integration, and this revealed clear differences between the treatment groups. While both control groups were severely and significantly impaired throughout the testing period, making many footslip errors as they traversed the ladder, LV-ChABC treated animals showed a marked recovery of function. Significant improvements were observed as early as 1 week post-injury (33.1 ± 3.5 footslip errors for LV-ChABC compared with 48.8 ± 3.5 and 47.1 ± 3.9 for LV-GFP and contusion only, respectively; $p < 0.01$ two-way RM ANOVA, Bonferroni's *post-hoc*; Fig. 3.7C). Importantly, these effects were sustained into chronic stages post-injury, with LV-ChABC treated rats showing significant improvements over both control groups throughout the testing period (footslip errors at 10 weeks post-injury: 14.6 ± 1.6 , 28.3 ± 2.8 , 29.7 ± 3 for LV-ChABC, LV-GFP and contusion only, respectively;

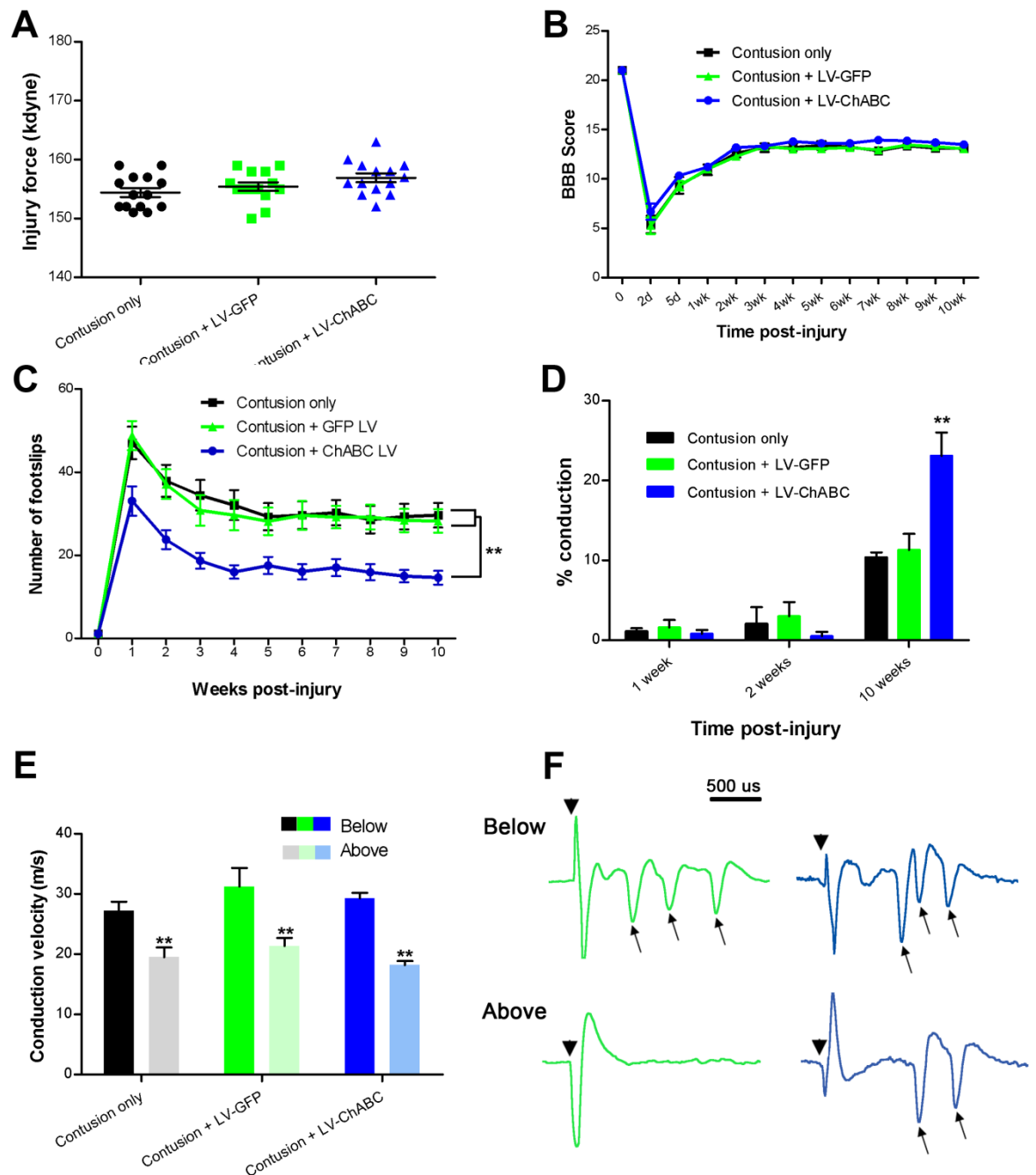


Figure 3.7. Gene delivery of ChABC leads to improved sensorimotor function and spinal conduction following spinal contusion. (A) Impact data showing the actual force applied to individual animals was within 10% of the intended force of 150 kdyne and mean values for each group were not significantly different ($n=14$ per group; $p>0.05$; one-way ANOVA), confirming that any group differences were not due to differences in the impact force during surgery. (B) Early Basso Beattie and Bresnahan (BBB) scores show a similar initial deficit in each group. Scores gradually improved over the first two weeks post-injury, but revealed no differences between treatment groups. (C) Treatment with LV-ChABC leads to an early and sustained

improvement in sensorimotor function, as shown by a significant reduction in footslips on the horizontal ladder task at all post-injury time-points compared to contusion only and contusion plus LV-GFP treatment (n=15 per group; $p<0.01$, two-way RM ANOVA, Bonferroni *post-hoc*). (D) Following treatment with LV-ChABC there is a significant improvement in the percentage of sensory dorsal column axons that are capable of conducting through the injury site at a chronic (10 weeks) post-injury time-point ($p<0.01$, one-way ANOVA, Tukey's *post-hoc*); this improvement in spinal conduction is not evident at early time-points (1 and 2 weeks) (n=5 per group for each time-point). (E) All groups display a similar slowing in conduction velocity when stimulating above the injury site. (F) Example traces recorded from dorsal root filaments (antidromic activation) at 10 weeks post-injury, whilst stimulating below and then above the lesion, give an example of multiple single units recorded through the injury site in a LV-ChABC treated animal (blue) and a complete lack of conduction through the injury site in a LV-GFP treated animal (green). Arrows identify single units of activity.

$p < 0.01$ two-way RM ANOVA, Bonferroni's *post-hoc*; Fig. 3.7C). The discrepancy between the two behavioural tasks perhaps reflects the different aspects of motor function assessed by each. While the BBB score is highly sensitive to small improvements in gross measures such as joint movements, weight support and stepping ability, it may be less sensitive to finer sensorimotor integration such as that measured by ladder walking.

Electrophysiology was then used to assess whether LV-ChABC could lead to improved conduction of spinal axons following contusion injury and intraspinal injection (Fig. 3.7D-F). Axonal conduction in long distance sensory projections in the spinal cord is severely and chronically impaired in this injury model, with only a small percentage of axons capable of conducting through the injury site (James et al., 2011). Single unit recordings of dorsal column axons antidromically-activated from above and below the T10 contusion injury revealed that, in the sub-acute injury stages, very few fibres were able to conduct across the injury site and no improvements in spinal conduction were observed following any treatment (percentage of conducting sampled fibres was $1.1 \pm 0.4\%$, $1.58 \pm 0.96\%$ and $0.8 \pm 0.49\%$ at 1 week post-injury and $2.07 \pm 2.07\%$, $3 \pm 1.78\%$ and $0.56 \pm 0.56\%$ at 2 weeks post-injury for contusion only, LV-GFP and LV-ChABC, respectively; $p > 0.05$ one-way ANOVA; Fig. 3.7D). However, at a chronic post-injury time-point (10 weeks), electrophysiological recordings of single unit activity across the lesion site revealed a potent effect of LV-ChABC treatment on restoring sensory fibre conduction following spinal contusion, with the percentage of conducting fibres in LV-ChABC animals significantly increased compared to animals with contusion only or contusion plus LV-GFP ($23.1 \pm 2.9\%$, compared to $10.4 \pm 0.6\%$ and $11.3 \pm 2\%$, respectively; $p < 0.01$ one-way ANOVA, Tukey's *post-hoc*; Fig. 3.7D).

Improved conduction was not associated with changes in conduction velocity, with mean conduction velocity of dorsal column sampled fibres significantly slower when stimulating above the injury than below in all groups (19.51 ± 1.61 , 21.35 ± 1.37 and 18.21 ± 0.64 versus 27.25 ± 1.47 , 31.22 ± 3.11 and 29.27 ± 0.91 m/s for contusion only, LV-GFP and LV-ChABC respectively; $p < 0.01$, paired t-test) and no significant differences in conduction velocity between groups ($p > 0.05$, one-way ANOVA; Fig. 3.7E). The slowing of conduction velocity through the injury site is likely due to factors such as demyelination and disrupted membrane properties (McDonald and Sears, 1970a; Tator and Fehlings, 1991) and suggests that although conduction is restored in a greater percentage of dorsal column fibres following LV-ChABC treatment, some aspects of conduction remain suboptimal. To determine whether LV-ChABC can affect axonal function without a lesion, percentage conduction was assessed in uninjured animals 8 weeks following intraspinal injection of LV-ChABC or LV-GFP. Normal conduction was observed in these animals, with no changes in the percentage of sampled fibres conducting across the injection site or the conduction velocity (Fig. 3.8). Thus, a single administration of LV-ChABC at the time of injury led to improved conduction of spinal axons at a chronic post-injury time point following spinal contusion, while no effect was noted even after an extended period with similar injections in the uninjured animal.

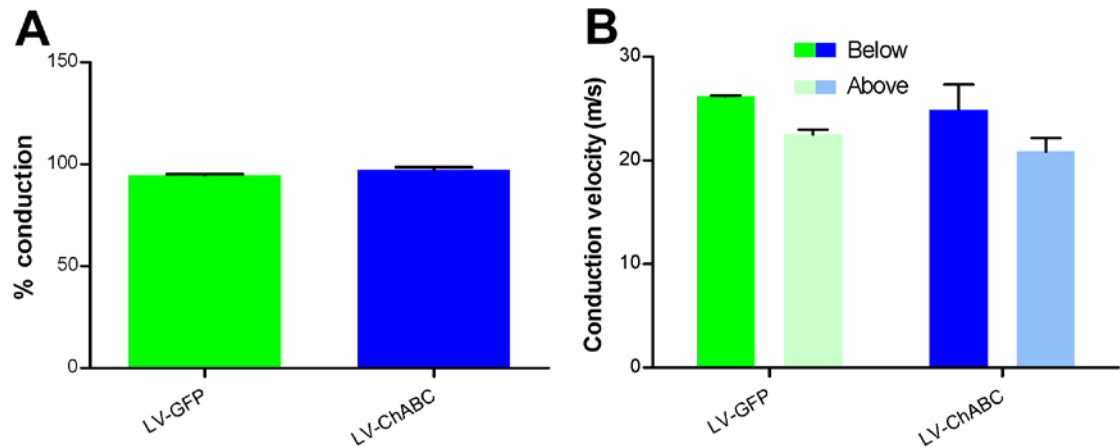


Figure 3.8. Treatment with LV-ChABC or LV-GFP has no effect on conduction in the uninjured spinal cord. (A) Intraspinal injection of either LV-GFP or LV-ChABC does not alter the extent of conduction through the injection site ($p > 0.05$, unpaired t-test). (B) Mean conduction velocity does not significantly change when stimulating above the injection site following treatment with LV-GFP or LV-ChABC ($p > 0.05$, paired t-test). The slight slowing of mean conduction velocity in both groups when stimulating above the injection site is due to the differences in conduction properties between the dorsal root and the spinal cord portions of the pathway (i.e. axons conduct faster in the dorsal root than in the spinal cord) and is also observed in naïve animals.

3.3.4 The effects of LV-ChABC on plasticity

ChABC treatment has previously been associated with enhanced plasticity and connectivity (Pizzorusso et al., 2002;Barritt et al., 2006;Cafferty et al., 2008;Alilain et al., 2011), so next it was determined whether large-scale CSPG digestion following LV-ChABC treatment would lead to functional plasticity of spinal reflexes below the injury following spinal contusion. Electrophysiology was used to examine the extent of wind up present when repeatedly activating hindlimb reflexes. Wind up of spinal reflexes normally only occurs when stimulating at an intensity sufficient to activate C-fibres. However, 12 weeks following spinal contusion, animals treated with LV-ChABC displayed wind up of two spinal reflexes (elicited by stimulation of the sural nerve whilst recording from the biceps femoris or common peroneal nerves) when stimulating at intensities sufficient to activate only A-fibres (Fig. 3.9A-F), indicating that long-term CSPG digestion leads to plastic changes in spinal connectivity. No such changes were apparent in control LV-GFP treated animals, indicated by a lack of wind up at any of the three A-fibre stimulus intensities (Fig. 3.9A-F). Whilst treatment with LV-ChABC led to significant changes in the wind up of spinal reflexes when activating only A-fibres, reassuringly there were no differences in C-fibre wind up between either of the treatment groups (Fig. 3.10). This suggests that the enhanced plasticity of spinal reflexes is unlikely to translate to the hypersensitivity of nociceptive function. Indeed, when behavioural assessments were carried out to determine withdrawal thresholds to thermal and mechanical stimulation of the paw and pain behaviour following formalin injection, there were no significant differences observed between LV-ChABC or LV-GFP treatment groups; this was apparent in both uninjured (Fig. 3.11) and injured (Fig. 3.12) animals, thus indicating that long-term CSPG digestion does not lead to enhanced pain sensitivity.

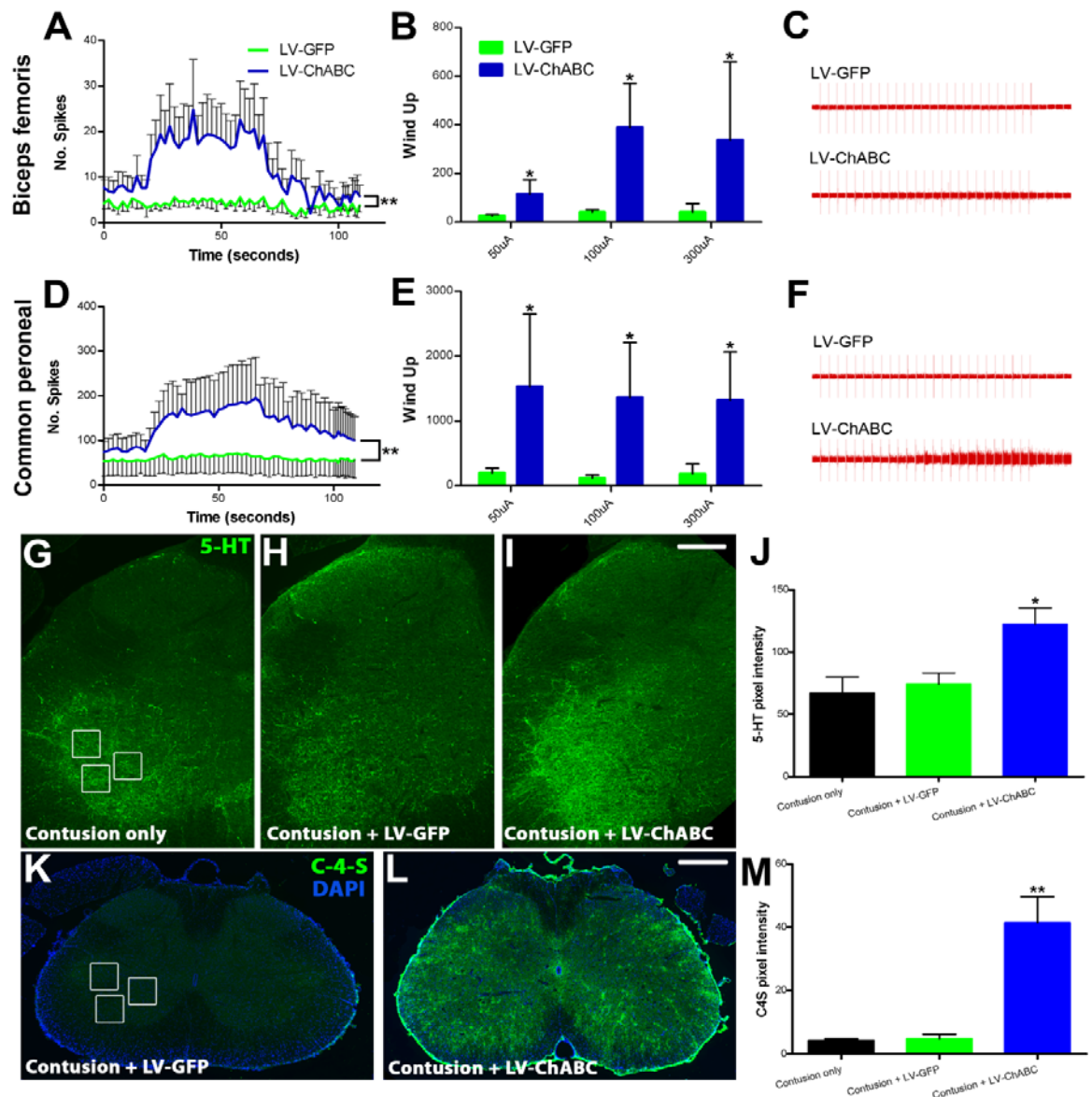


Figure 3.9. Gene delivery of ChABC leads to plastic changes in spinal circuitry following spinal contusion. (A+D) Quantification of total spikes recorded every 2 seconds before (0-20s), during (20-70s), and after (70-120s) repeated stimulation of the sural nerve at 50 μ A, from the main nerve innervating the biceps femoris (BF, **A**) and the common peroneal (CP, **D**) nerves. Animals treated with LV-ChABC show a gradual increase in the excitability of this reflex following onset of stimulation, whilst those treated with LV-GFP remain unaffected (n=4 per group; $p < 0.001$, two-way RM ANOVA). (**B+E**) Quantification of the total increase in excitability during stimulation compared to response to the first stimulus (wind up) at three different low-threshold stimulation intensities (i.e. no activation of C-fibres) for the BF (**B**) and the CP (**E**), indicating that treatment with LV-ChABC leads to a significant enhancement in the

excitability of these reflexes at all three stimulation intensities (n=4 per group; $p<0.05$, unpaired t-test). **(C+F)** Example traces recorded from BF **(C)** and CP **(F)**, illustrating the enhanced excitability observed in each nerve following repeated stimulation of the sural nerve at 50 μ A in LV-ChABC treated, but not LV-GFP treated, animals. **(G-J)** Serotonergic sprouting is significantly enhanced in the ventral horn of lumbar spinal cord (L5) following treatment with LV-ChABC **(I)** compared to contusion only **(G)** and LV-GFP **(H)** (quantified in **J**; n=4 per group; $p<0.05$, one way ANOVA, Tukey's *post-hoc*). **(K-M)** C-4-S immunoreactivity is abundant in the lumbar cord (L5) of LV-ChABC treated animals **(L)**, but absent in both LV-GFP and contusion only animals **(K+M)** ($p<0.001$, one-way ANOVA, Tukey's *post-hoc*); boxes in **(G)** and **(K)** show regions where intensity measurements were quantified. **G, H, I**, Scale bar = 250 μ m; **K, L**, Scale bar = 500 μ m.

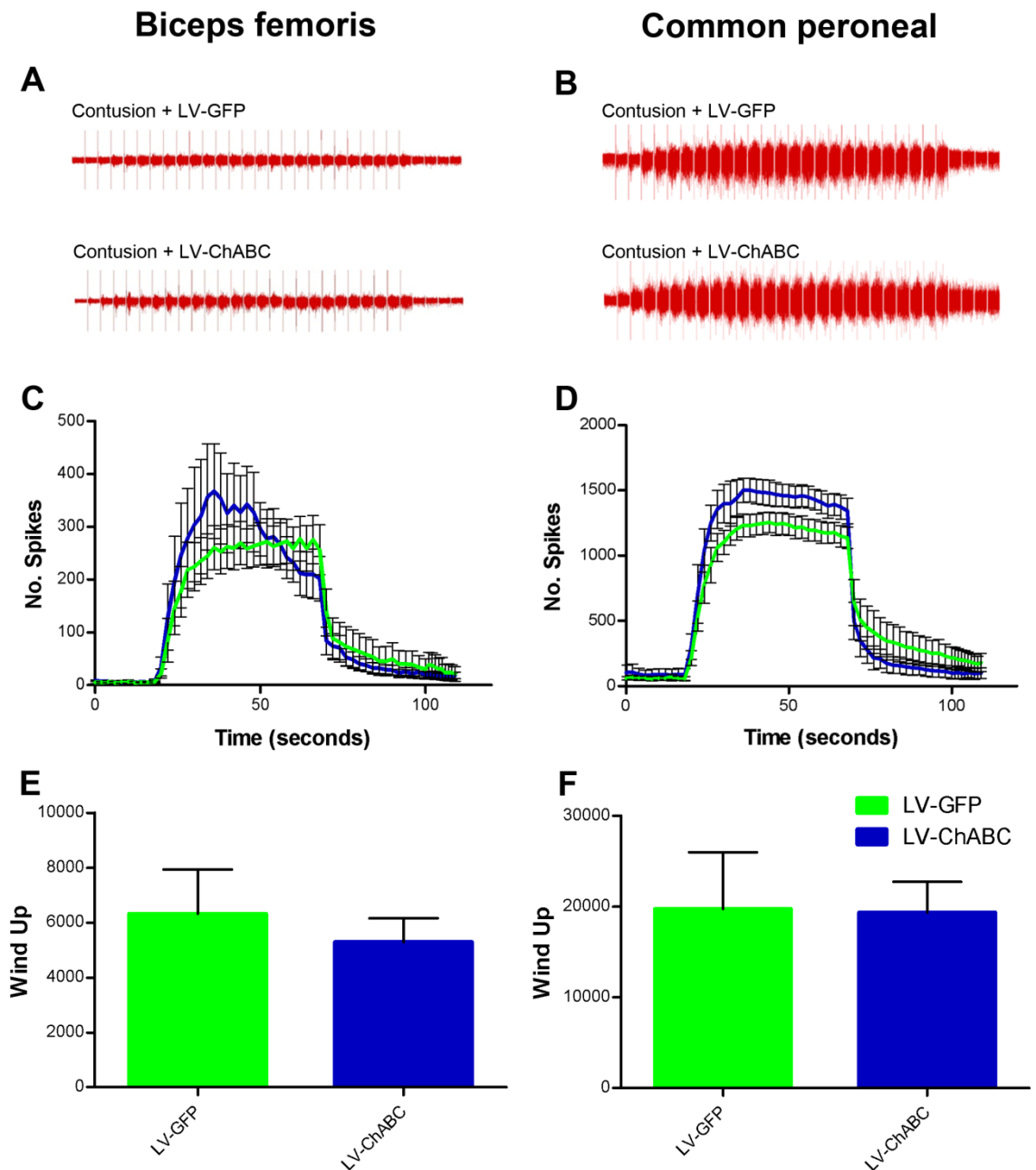


Figure 3.10. C-fibre wind-up of hindlimb reflexes is induced to the same extent in LV-GFP and LV-ChABC treated animals. (A+B) Example traces of the wind-up induced in the biceps femoris (BF, **A**) and the common peroneal (CP, **B**) nerves following repeated stimulation of the sural nerve at 4 mA. (C+D) Quantification of the total spikes recorded from the BF (**C**) and the CP (**D**) every 2 seconds before (0-20s), during (20-70s), and after (70-120s) stimulation. (E+F) Quantification of the total wind-up occurring in the BF (**E**) and the CP (**F**).

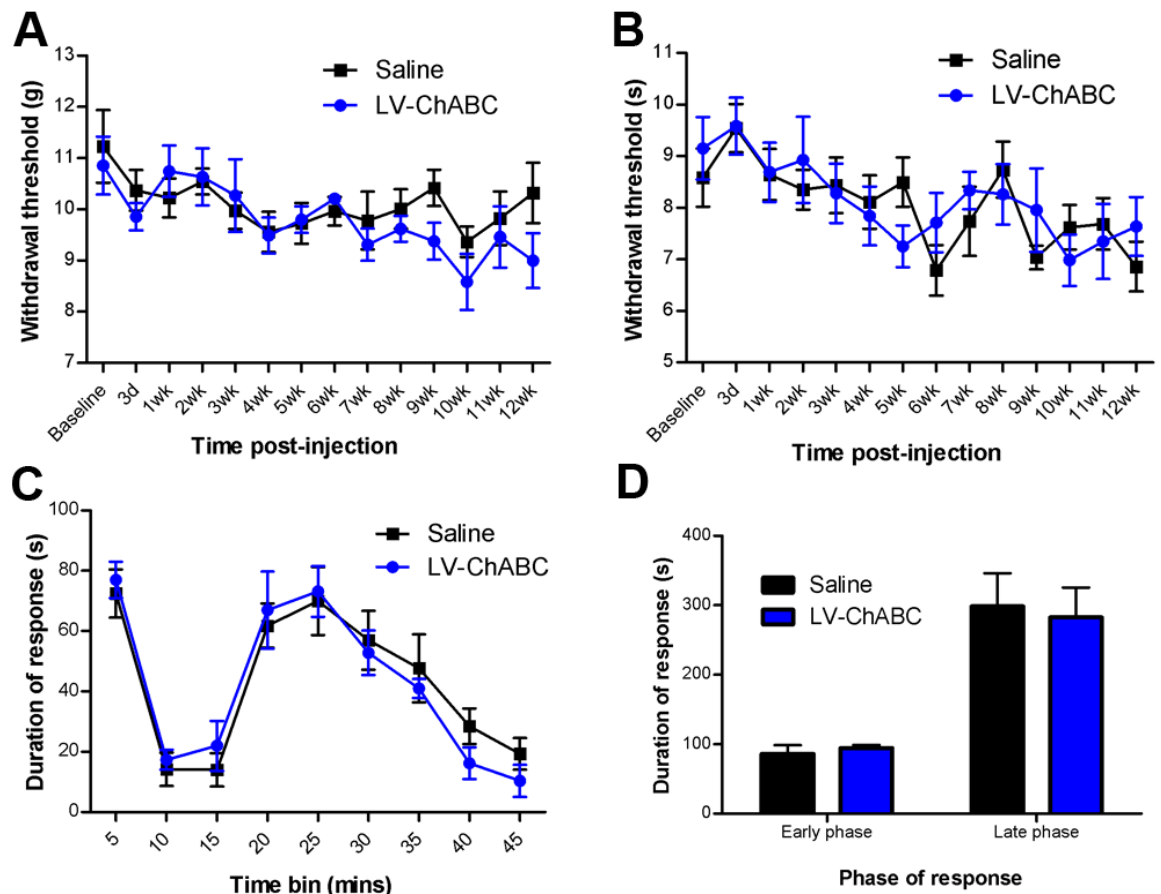


Figure 3.11. Long-term CSPG digestion does not lead to an enhanced pain state in uninjured animals. (A+B) Assessments of withdrawal thresholds to mechanical (von Frey test) (A) or thermal (Hargreaves test) (B) stimulation of the forepaw following injection of saline or LV-ChABC into the C5 spinal cord revealed no significant changes in pain sensitivity over a 12 week time course. (C+D) Injection of formalin to elicit a pain response resulted in typical biphasic licking and biting behaviours in the affected forepaw, with no significant differences observed between groups during the early or late phases of the formalin-evoked response, indicating no enhanced sensitivity to pain following long-term treatment with LV-ChABC (n=7/8 per group; $p>0.05$ two-way RM ANOVA for time course data and Student's t-test for early and late phase data).

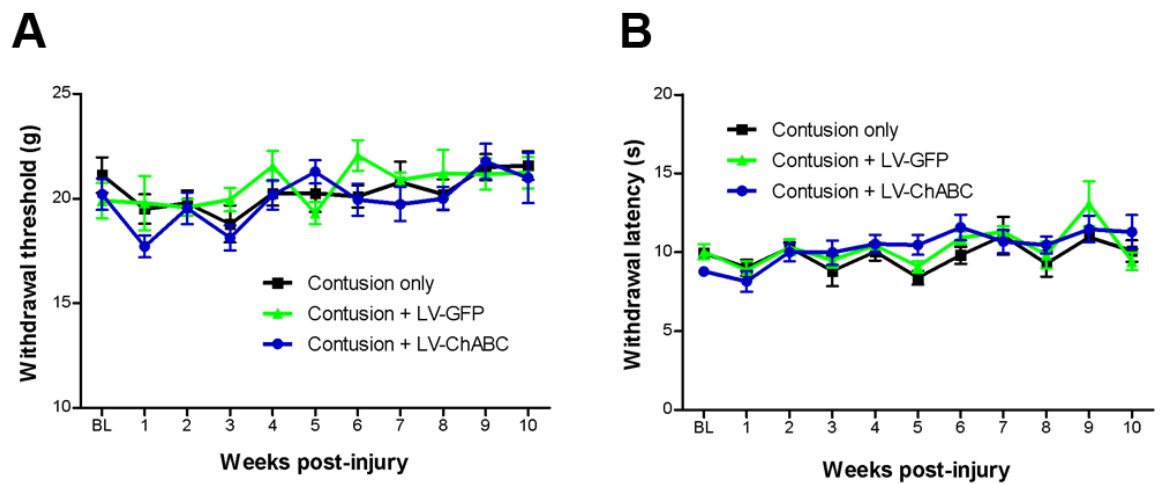


Figure 3.12. Chronic delivery of ChABC does not lead to an enhanced pain state following spinal contusion. (A+B) Assessments of withdrawal thresholds to (A) mechanical (von Frey test) or (B) thermal (Hargreaves test) stimulation of the hindpaw following T10 spinal contusion and injection of saline or LV-ChABC at the injury site revealed no significant changes in pain sensitivity over a 10 week time course (n=7/8 per group; $p>0.05$ two-way RM ANOVA).

Serotonergic projections that descend from the raphe nuclei and terminate in the ventral horn of the spinal cord provide excitatory input to motoneurons, the loss of which correlates with locomotor dysfunction (Saruhashi et al., 1996). To determine whether CSPG digestion with LV-ChABC could lead to increased serotonergic input onto targets caudal to the injury, 5-HT immunoreactivity was examined in the ventral horn of the lumbar spinal cord 12 weeks following contusion and intraspinal injection. Dense serotonergic innervation was apparent in the ventral horn of LV-ChABC treated animals, in contrast to low 5HT-immunoreactivity in the ventral horn of animals with contusion only or contusion plus LV-GFP, (Fig. 3.9G-I), with a statistically significant increase in serotonergic fibre density (mean pixel value of 122.2 ± 13.1 compared to 67.03 ± 12.7 and 74.13 ± 9.1 , respectively; $p < 0.05$ one-way ANOVA; Fig. 3.9J). Corresponding to increased serotonergic input, intense C-4-S immunoreactivity was observed in the lumbar spinal cord of LV-ChABC treated animals, which was absent in contusion only or GFP-treated animals (C-4-S mean pixel value 41.35 ± 8.35 , compared to 4.28 ± 0.53 and 4.86 ± 1.36 , respectively; $p < 0.001$ one-way ANOVA; Fig. 3.9K-M). These findings suggest that widespread CSPG digestion achieved with LV-ChABC can promote changes in anatomical and functional connectivity below the level of a SCI.

3.3.5 LV-ChABC alters the immune response to SCI

Local and infiltrating macrophages are key effectors of tissue remodelling and repair following injury. Since the data described indicated that gene delivery of ChABC led to a significant improvement in tissue preservation, the effect of LV-ChABC treatment on macrophage response was assessed using immunohistochemistry firstly at 1 week-post injury. Following observations of a dramatic upregulation of CD206 at the injury site in

LV-ChABC-treated animals, the main marker of alternative (M2) macrophage activation, particularly evident in comparison to untreated animals (Fig. 3.13A+B), further analysis was carried out at 3 day and 2 week post-injury time points. Interestingly, clusters of CD206 positive cells were apparent around the lesion edges in LV-ChABC animals, particularly in areas where the glial scar typically forms by 10 weeks post-injury (Fig. 3.13C+D), suggesting a potential underlying mechanism for observed changes in reactive gliosis in LV-ChABC-treated animals. The phagolysosomal marker CD68 was notably increased at the injury site of LV-ChABC treated animals 3 days after injury in comparison to untreated animals (Fig. 3.13E+F). CD206 levels remained high in LV-ChABC treated animals at 2 weeks post-injury, whilst there were still remarkably low levels of this marker for M2 macrophage activation in untreated animals (Fig. 3.13G+H). Furthermore, co-localisation of IBA1 with CD68 and CD206 at the lesion site indicated that macrophage/microglia numbers (identified by IBA1-immunopositive cells) within the lesion epicentre were similar regardless of treatment (Fig. 3.13E-H).

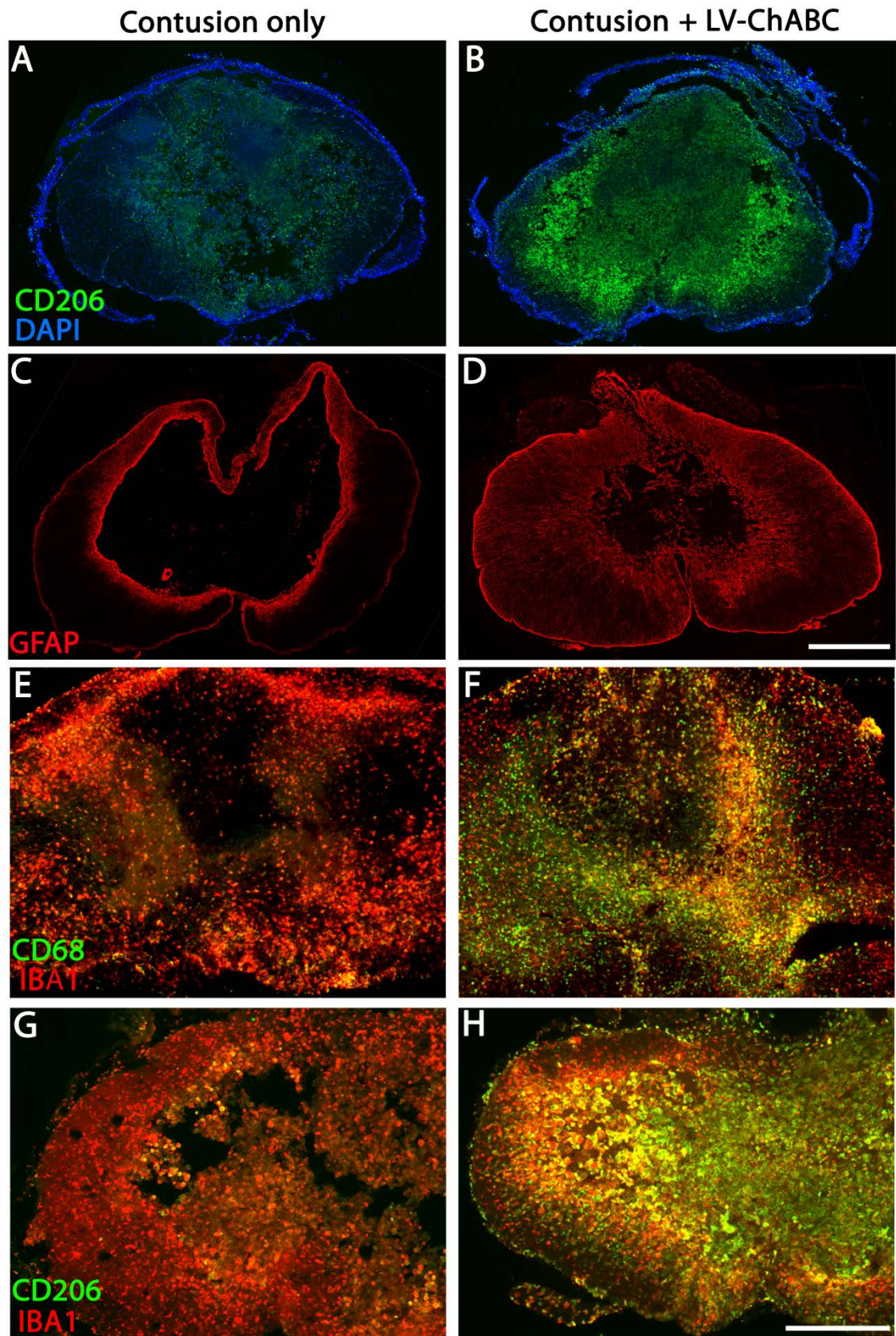


Figure 3.13. Gene delivery of ChABC leads to alterations in macrophage response. At 1 week post-injury LV-ChABC treatment is associated with increased CD206 immunoreactivity

(**A**) (green, a marker for M2 macrophages) compared to contusion only (**B**). Areas of intense CD206 immunoreactivity correspond to areas where the glial scar typically forms by 10 weeks post-injury (**C+D**). Double labelling with IBA1 (macrophages/microglia, red) shows the early increase in CD68 immunostaining (green, a marker for phagolysosomal cells) at the injury epicentre at 3 days post-injury in LV-ChABC treated animals (**E**) compared to contusion only controls (**F**). Levels of CD206 immunostaining (green) remain highly elevated at 2 weeks post-injury in LV-ChABC treated animals (**G**), but remain remarkably low in untreated animals (**H**). Scale bar, 500µm.

3.4 Discussion

The results of this study demonstrate that chronic delivery of genetically modified ChABC, via viral vector gene transfer to spinal cord neurons, can prevent much of the secondary degenerative pathology that occurs following SCI, in an experimental model that closely mimics human traumatic SCI. Improved pathology was associated with enhanced behavioural recovery, improved spinal conduction and plasticity of spinal reflexes. In addition, an association between large-scale CSPG digestion and modulation of macrophage phenotype to favour a reparatory inflammatory response was observed, revealing a novel potential mechanism underlying repair.

3.4.1 Long-term gene expression using a lentiviral vector

Chronic expression of ChABC has not previously been shown. Due to issues of enzyme stability, ChABC has typically been administered via repeated bolus injections into indwelling catheters placed intrathecally at the lesion site (Bradbury et al., 2002;Caggiano et al., 2005;Garcia-Alias et al., 2009a), in the intracerebroventricular system (Carter et al., 2008) or via multiple injections directly into CNS parenchyma (Pizzorusso et al., 2002;Massey et al., 2006). Thermostablising the enzyme can prolong the activity of ChABC and effectively reduce CSPG levels at lesion boundaries for 6 weeks following one administration (Lee et al., 2010a), although delivery remains restricted to the injection site in contrast to the widespread CSPG digestion observed after LV-ChABC delivery. Several studies have observed long-term expression of a transgene following direct vector injection into the spinal cord, with vector-driven expression of trophic factors observed in spinal neurons for 3-4 months in ventral root avulsed rats (Blits et al., 2004) and in mouse models of amyotrophic lateral sclerosis

(ALS) (Guillot et al., 2004). In this chapter a single intraspinal injection of LV-ChABC enabled efficient delivery of active enzyme for at least 1 year. This is not only important for developing therapeutics for SCI, but also has implications for other disorders, such as ALS and multiple sclerosis (MS), where long-term delivery to large areas of the spinal cord may be advantageous.

3.4.2 LV-ChABC leads to functional improvements

The consequences of large-scale CSPG digestion have not previously been assessed. A significant functional recovery on the horizontal ladder following LV-ChABC treatment was observed, which is likely due to early neuroprotective effects since these animals never reached the same level of impairment as the other groups on this task, despite having the same severity injury. This is in line with the histological observations of tissue preservation and the reduced progression of secondary injury pathology following LV-ChABC. However, despite the significant improvements in ladder walking ability and spinal conduction, the lack of effect on BBB locomotor scoring suggests a limit to the observed recovery following LV-ChABC treatment. This also highlights one of the potential issues with the BBB scoring system, specifically the non-linearity of the scoring system (Basso et al., 1995). During BBB scoring sessions various aspects of hindlimb locomotor function are assessed during open field locomotion, but the ability of the BBB score to reflect many of these functions depends on an animal achieving specific milestones in its locomotor recovery. For example, if an animal does not achieve consistent forelimb/hindlimb coordination during its 4 minute assessment, then many aspects of locomotor function such as paw position, extent of toe clearance during stepping and trunk instability will contribute nothing to the BBB score it is assigned. Similarly, if specific patterns of paw placement are not achieved then toe clearance,

trunk instability and tail position will not be reflected in the BBB score. This means that, whilst the lower end of the BBB scale (<11) is sensitive to minor changes in function, any change in the upper end of the scale (≥ 13) can potentially reflect a far greater improvement in function and, therefore, can be far more difficult to achieve. Interestingly, many of the studies in which an experimental treatment leads to improved BBB scores involve the use of fairly severe injury models, particularly in the case of contusion injury studies, and improvements are made in the lower end of the BBB scale (Caggiano et al., 2005; Pedram et al., 2010; Figueroa et al., 2012; Lu et al., 2012). The utilisation of a more severe contusion injury model may, therefore, reveal an effect of LV-ChABC on BBB locomotor scores and this will be investigated in chapter four.

The effect of chronic CSPG digestion on restoring the conduction of spinal axons was also assessed. The observed improvement in conduction of dorsal column axons through the injury site at a chronic (10 week) post-injury time point is likely to be due primarily to the significant sparing of spinal tissue following treatment with LV-ChABC, although it cannot be ruled out that there may have been some regeneration of dorsal column axons that could also account for improved conduction. The tracing of fibres in this pathway would have been particularly beneficial in aiding to elucidate the potential contribution of regeneration to improved functional outcomes. Specifically, pre-injury anterograde tracing of the dorsal column axons followed by post-injury retrograde tracing would allow a clear assessment of which fibres had truly regenerated and which had simply been spared; a distinction which is particularly important in an incomplete injury such as this. Whilst attempts at tract tracing in this study were unsuccessful, it would be of great interest as a future experiment to achieve successful tracing of both ascending and descending spinal pathways and assess axonal integrity and regeneration in this experimental paradigm. Alleviation of CSPG-induced

conduction block is also likely to be contributing to enhanced levels of conduction, since CSPGs have previously been shown to block conduction following SCI (Hunanyan et al., 2010). That axons conducting through the injury site in LV-ChABC treated animals do so at the same mean velocity as those in the control groups suggests that LV-ChABC is unlikely to be having an effect on remyelination, as this would lead to improvements in conduction velocity (McDonald and Sears, 1970a). This would also explain the lack of an effect of LV-ChABC at the sub-acute (1 and 2 week) time points, as axons in the dorsal columns will still be demyelinated at this stage, causing conduction block (McDonald and Sears, 1969b;James et al., 2011). Improved conduction in chronic stages also provides further evidence that sustained CSPG digestion may be important for return of spinal conduction following the early periods of acute demyelination and spontaneous remyelination that occur following spinal contusion.

3.4.3 LV-ChABC leads to plasticity below the injury

Plasticity of intact spinal pathways is known to occur spontaneously following injury to the spinal cord and can lead to the formation of compensatory spinal circuitry (Weidner et al., 2001;Bareyre et al., 2004;Rosenzweig et al., 2010) and enhancing this using experimental therapeutics is an important goal of SCI research. ChABC treatment has repeatedly been shown to significantly enhance this process (Barritt et al., 2006;Massey et al., 2006;Cafferty et al., 2008;Tom et al., 2009a;Alilain et al., 2011;Starkey et al., 2012). The finding that LV-ChABC treatment led to significant sprouting of serotonergic fibres was therefore in agreement with these previous studies. The underlying mechanisms responsible for compensatory sprouting after injury are not altogether clear. As spontaneous sprouting does not occur in the intact CNS, some of the

physiological changes caused by injury must be responsible for the sudden stimulation of axonal sprouting. A logical potential mechanism would be that the loss of input to many cells stimulates them to secrete a number of growth promoting molecules in order to encourage reconnection. Indeed, a recent study showed that following unilateral injury to the CST, inhibition of trk-B signalling in the intact CST prevented compensatory sprouting towards the denervated side (Ueno et al., 2012). This suggested that BDNF release from the denervated side of the spinal cord, perhaps from denervated interneurons, may be responsible for the commissural sprouting of intact CST fibres. This hypothesis was backed up by evidence showing that by reducing BDNF release from the denervated grey matter, compensatory sprouting of the intact CST could be significantly attenuated (Ueno et al., 2012). If the release of growth factors from denervated cells is indeed a key molecular mechanism underlying compensatory sprouting, then this would also provide a feasible explanation as to why ChABC enhances spontaneous plasticity; CS-GAGs are known to bind various growth factors (Deepa et al., 2002; Nandini et al., 2004; Nandini and Sugahara, 2006) and therefore may competitively bind to any growth factors released by denervated cells. ChABC-mediated removal of CS-GAGs prevents this interaction and any endogenously released growth factors will be able to freely interact with neuronal targets.

Whilst the effects of LV-ChABC on serotonergic sprouting in this study were expected and in line with previous findings (Barritt et al., 2006; Tom et al., 2009a; Karimi-Abdolrezaee et al., 2010; Alilain et al., 2011), the finding that LV-ChABC altered the excitability of spinal reflexes following A fibre activation was a novel observation. This finding suggests there have been plastic changes in the organisation of spinal circuitry and/or synaptic composition. Interestingly, administration of ChABC to the cervical spinal cord has also been shown to induce

plasticity of forelimb reflexes following injury of forelimb peripheral nerves (Bosch et al., 2012). The disruption of PNNs due to CSPG degradation enhances synaptogenesis (Pizzorusso et al., 2002;Berardi et al., 2004). Sprouting fibres, such as the serotonergic fibres observed in this study, are more likely to form *de novo* connections in the absence of inhibitory PNNs and, therefore, enhanced excitability could result from an increased number of synaptic inputs on dorsal horn interneurons or ventral horn motor neurons. Equally the observed functional plasticity could be due to changes at the synaptic level. Degradation of PNNs surrounding hippocampal neurons in culture has resulted in enhanced short-term synaptic plasticity, as shown by a significant decrease in paired-pulse depression (Frischknecht et al., 2009). This change was due to increased exchange between synaptic and extra-synaptic AMPA receptors, indicating that degradation of PNNs led to enhanced receptor mobility. Changes in the levels and exchange rates of synaptic receptors could well have contributed to the plasticity of spinal reflexes observed following LV-ChABC treatment. If the observed changes in spinal reflex excitability are solely caused via this mechanism then it would be expected that LV-ChABC would have the same effect in the uninjured cord, this will therefore be an interesting experiment to carry out in the future.

3.4.4 The importance of widespread CSPG degradation

Enhancing plasticity of lumbar spinal circuits following thoracic spinal cord injury is an important mechanism for stimulating functional repair (van den Brand et al., 2012). Observations of *de novo* plasticity of spinal reflexes below the injury following LV-ChABC treatment provide strong evidence supporting the hypothesis that CSPGs restrict the capacity for reorganisation of spinal connections (Pizzorusso et al., 2002;Massey et al., 2006;Cafferty et al., 2008) and that large-scale CSPG digestion can

lead to functional plasticity at sites distal to the injury, a finding substantiated by the observations of increased serotonergic input in regions containing heavily digested CSPGs. Widespread delivery therefore appears to be important, both for eliciting plasticity at sites distal to the injury and for improving conduction in long-range spinal projections. Widespread CSPG digestion may also be important for the immunomodulatory effects of LV-ChABC, since activated macrophages typically spread over several spinal segments following contusion injury (David and Kroner, 2011), likely requiring treatment of a large area to elicit significant macrophage modulation.

3.4.5 Immunomodulation as a potential mechanism for repair

The local inflammatory response following SCI is known to be neurotoxic and inhibitory to regeneration (Silver and Miller, 2004). Previous work has shown that classically activated M1 macrophages are rapidly induced and maintained at sites of traumatic SCI, followed by a smaller and transient M2 macrophage response (Kigerl et al., 2009; David and Kroner, 2011). *In vitro* and *in vivo* evidence suggests that M1 macrophages are neurotoxic (Kigerl et al., 2009) while M2 macrophages promote resolution of inflammation, angiogenesis and tissue remodeling and exhibit enhanced phagocytic properties following injury (Mantovani et al., 2002). Polarizing macrophages towards an M2 phenotype may therefore limit secondary inflammatory-mediated injury (Kigerl et al., 2009). This study has shown that gene delivery of ChABC induced expression of CD206, a well-characterized marker of M2 macrophage polarization, in macrophages in the lesion epicentre 1 and 2 weeks post-injury. The CD206 positive macrophages were not always associated with IBA1 immunostaining, especially in the white matter and lesion core, indicating that these cells were not

exclusively derived from local activated microglia. Interestingly, Shechter *et al* recently demonstrated that infiltrating monocyte-derived macrophages enhanced tissue recovery and repair following SCI in mice and that these trafficking monocytes have M2 phenotypic characteristics (Shechter et al., 2013). The upregulation of CD206 in LV-ChABC treated cords was preceded by an early and transient increase in CD68, a lysosome membrane protein whose expression and post-translational modification is increased in actively phagocytic macrophages (Damoiseaux et al., 1994;da Silva and Gordon, 1999). Early phagocytosis of apoptotic cells and myelin debris could enhance repair and correlates well with the tissue preservation and neuroprotection that we observed in LV-ChABC treated cords. It is possible that the ability of alternatively activated macrophages to promote resolution of inflammation, clearance of cytotoxic debris and positive tissue remodelling provides a mechanism underlying the significant tissue sparing and neuroprotection that were observed following gene delivery of ChABC in the contused spinal cord.

3.4.6 Summary

The findings of this chapter demonstrate that using a gene delivery vector-based approach enables resident CNS cells to stably secrete ChABC *in vivo* for a significant period of time with a single injection delivery. Furthermore, this results in potent effects on reducing secondary injury pathology and restoring function in a clinically relevant model of traumatic spinal contusion. Finally, treatment with LV-ChABC appears to have modulatory effects on the immune response, activating a more reparatory immune phenotype that is likely to contribute to the observed neuroprotection. Early CSPG digestion appears to be important for immunomodulation and tissue preservation whilst sustained ChABC delivery is important for the return of spinal conduction and

enhancing plasticity of spinal circuits in distal spinal segments. These findings have widespread implications for the treatment of spinal injuries and other pathological disease states.

Chapter Four

4 Chapter 4:

Assessing the therapeutic potential of chondroitinase ABC gene therapy in contusion injury models of differing severity and spinal level

4.1 Introduction

SCI is a particularly heterogeneous clinical condition. Whilst any trauma to the cord is likely to lead to functional impairment, the ensuing profile of symptoms can vary dramatically depending of factors such as injury location and severity. Whilst SCI involving contusion of the cord is the most common form of injury (Norenberg et al., 2004), a contusion injury occurring in the mid-cervical region of the spinal cord will present a number of different symptoms to those which would be observed following a contusion injury in the mid-thoracic region of the spinal cord (Steeves et al., 2011; Zariffa et al., 2011). Equally, the severity of an injury has an obvious effect on the post-injury presentation of symptoms, ranging from complete loss of sensory and motor function below the level of the lesion to only minor deficits in one or the other. The range of symptoms associated with severity of injury is reflected by the existence of the AIS, which is used to classify the extent of neurological impairment in SCI patients (described in more detail in chapter one, section 1.1). The heterogeneity of SCI's, both in terms of injury severity and level of injury, highlights the importance of assessing the

efficacy of any potential therapeutic intervention in a variety of injury models; this will be discussed in greater detail below.

4.1.1 Importance of modelling injury at different spinal levels

The level at which the spinal cord is injured obviously leads to varying effects due to the fact that function is disrupted below the level of the injury, however the location of SCI can also lead to differential effects on the pathophysiological response. For example, the neuronal cell body response to injury is altered depending on whether axotomy occurs proximally or distally. Both rubrospinal and corticospinal neurons have been shown to significantly up-regulate regeneration-associated genes, such as GAP-43 and c-Jun, if axotomy occurs proximal to the cell body (Fernandes et al., 1999; Mason et al., 2003), but this response becomes far less pronounced the more distally axotomy occurs. Although proximal axotomy is associated with enhanced up-regulation of RAGs, increased proximity of axotomy also leads to increased levels of cell death (Tetzlaff et al., 1994; Fernandes et al., 1999; Hains et al., 2003; Liu et al., 2003). It is clear, therefore, that the location of an injury will differentially affect individual populations of neurons, with cervical injuries potentially causing more apoptosis of supraspinal neurons than a thoracic injury for example. In addition to the response of supraspinal neuronal cell bodies, neuronal cell bodies in the spinal grey matter will also respond differently to the location of injury; particularly if the location of the injury results in them being damaged by the primary trauma. Injuries at some spinal cord levels are likely to be more disruptive to important spinal neuronal populations than others. For example, the lumbar and cervical enlargements of the spinal cord contain important second order neurons and motor neuron pools vital for motor and sensory function of the limbs (Peyronnard and Charron, 1983; Vinay et al., 2000). The phrenic nuclei, which are vital for respiratory function, are also located bilaterally in the cervical

spinal cord (Berger, 1979; Alilain et al., 2011; Dougherty et al., 2012; Lane et al., 2012). Damage to important spinal neuronal populations in the grey matter (associated with respiratory and/or upper limb function) and disruption of axonal pathways in the white matter are both important contributing factors to loss of function following injury at cervical level. The disruption of function caused by neuronal cell body damage following a thoracic injury is far less pronounced however, as these cell bodies primarily mediate the function of postural trunk muscles, meaning that damage to white matter is likely to be the primary cause of functional impairment.

The differences in response to SCI due to injury level means that injuries at some spinal cord levels may respond better to certain therapeutic strategies than injuries at other levels. For example, neuroprotective strategies resulting in increased neuronal survival at the injury site would likely lead to highly significant functional benefits following a cervical injury, due to the importance of neuronal cell bodies located here in respiratory or upper limb function. The enhanced survival of neuronal cell bodies following thoracic injury would likely result in far less noticeable improvements in function. However, significantly increased white matter sparing at either injury level would be expected to be equally effective. Additionally, regeneration over relatively short distances following cervical injury could result in successful reinnervation of brainstem nuclei and/or important motor neuron pools. For example, following a C2 hemisection, treatment with a peripheral nerve graft and ChABC to encourage regeneration as well as degrade PNNs surrounding phrenic motor neurons was shown to result in reinnervation of the ipsilateral phrenic nucleus (located C3-C6) and significantly improve respiratory function (Alilain et al., 2011). The restoration of respiratory function in this study effectively represented a change of injury level from C2 to C4, at least in terms of respiratory function. In animal models of injury the

functional benefit of a therapeutic which led to the lowering of injury level may go undetected if assessed in a thoracic injury, as the lowering of an injury by a few segments at thoracic level may not result in obvious improvements. This emphasises the importance of assessing potential therapeutics in SCI models at different spinal levels.

In addition to the importance of modelling SCI at different levels, cervical injury models are particularly important in regard to clinical relevance. Just over half of all human SCIs occur at cervical level, making it the most common injury level (followed by thoracic injury which represents ~35% of SCI patients; www.nscisc.uab.edu) (Norenberg et al., 2004; Jackson et al., 2004; Anderson et al., 2009b). Over a quarter of all spinal injured patients have suffered from a contusive injury at the cervical level (Pearse et al., 2005), making cervical contusion models the most representative and clinically relevant pre-clinical models of injury. In addition to their clinical relevance, cervical injury models also allow the study of impairments in respiratory and/or upper limb function (Moreno et al., 1992; Li et al., 2003; Dunham et al., 2010; Alilain et al., 2011; Khaing et al., 2012), functions which are of the highest priority to SCI patients (Anderson, 2004; Snoek et al., 2005; Wagner et al., 2007; Simpson et al., 2012).

4.1.2 Importance of modelling injuries of different severities

As is the case with the spinal level at which a SCI occurs, the severity of an injury also has important implications for the subsequent pathophysiological response. Obviously the more severe a trauma to the spinal cord is, the more damage it will cause. However, the effects of injury severity are more complex than just the extent of initial damage caused. For example, increased injury severity will also lead to more extensive demyelination (Pearse et al., 2005; Ghasemlou et al., 2005; Nishi et al., 2007), increased microglial and astroglial activation (Farooque et al., 1995; Ghasemlou et al., 2005) reflecting an increased inflammatory response and glial scarring, and graded changes in

levels of molecules such as amino acids (Farooque et al., 1996) and ions such as sodium, potassium and calcium (Kwo et al., 1989) at the injury site. Furthermore, differences in injury severity result in differing patterns of damage in terms of which spinal pathways and neuronal populations in the spinal cord become impaired (Soblosky et al., 2001). This graded effect of injury on spinal pathways and neuronal populations is an important consideration when assessing potential therapeutics, as many experimental therapies have been shown to elicit different responses in different spinal tracts (Giehl and Tetzlaff, 1996; Hammond et al., 1999; Blesch et al., 2004; Kwon et al., 2004; Blesch et al., 2012a). For example, infusion of the injury site with exogenous BDNF has been shown to promote sprouting of both rubrospinal and raphespinal tracts (Kobayashi et al., 1997; Ye and Houle, 1997), but have little effect on sensory axons in the dorsal funiculi (Bradbury et al., 1998; Bradbury et al., 1999). It can therefore be beneficial to assess novel therapeutics injury models of different severity in order to gain a better understanding of their effects, as some effects may only become apparent at certain injury severities. For instance, treatment of a “severe” thoracic contusion injury with intrathecal infusions of ChABC enzyme every other day resulted in significant improvements in BBB locomotor scores, whilst the same treatment regime used following a “mild” thoracic contusion had no effect on BBB scores at all (Caggiano et al., 2005). As there is such a wide scope for injury severity and level in SCI patients, ideally any potential treatment for SCI moving forward to clinical trials should have shown efficacy at a number of different severities and spinal levels in preclinical injury models. Alternatively, different injury types in SCI patients could be targeted using different treatment types that have been shown to be particularly efficacious in specific injury types.

4.1.3 Assessing the efficacy of LV-ChABC following spinal contusion at different injury levels and severity

Having assessed the efficacy of LV-ChABC in a moderate thoracic contusion injury in the previous chapter, this chapter aims to further assess the therapeutic potential of LV-ChABC using both a moderate cervical contusion injury and a more severe thoracic contusion injury than that used in the preceding chapters. Firstly, a model of cervical contusion injury was optimised by assessing post-injury functional changes using a variety of behavioural tests including locomotor rating, the horizontal ladder, forelimb grip-strength, the inclined plane, a sticky tape removal task and a hanging test. Additionally a variety of gait parameters were assessed using the Digigait gait analysis system. This was carried out in both male and female rats in order to evaluate which would better tolerate the injury and if there would be any major differences between the two sexes in their response to injury. The efficacy of LV-ChABC was then assessed using this injury model in male rats and the most optimal behavioural tests. LV-ChABC treatment was found to significantly improve skilled locomotor function and conduction of long-distance sensory axons through the lesion site, as well as leading to mild improvements in forelimb grip-strength and corticospinal tract function. Histological analysis of cavity formation showed that LV-ChABC treated animals developed the smallest cavity at lesion epicentre and the rostro-caudal spread of cavitation was reduced. Finally, a smaller study was performed in order to assess the effects of LV-ChABC on locomotor function and sensory conduction following a severe thoracic contusion injury. Whilst there were no detectable improvements in locomotor function using the BBB locomotor scale, LV-ChABC again lead to improved conduction of sensory axons through the lesion site in the injury model and this was associated with improved tissue preservation.

4.2 Materials and methods

4.2.1 Animals and study design

Adult male and female Sprague Dawley rats (Harlan laboratories) were used (housed under a 12 hour light/dark cycle with free access to food and water). The experiments described in this chapter can be separated into three separate studies:

Study one: Optimisation of a moderate cervical contusion injury model (225 kdyne at C5). This was originally carried out on adult female rats (200-250g), but mortality rates were high (beginning with n=10, but only n=5 surviving, mortality rate of 50%), so this was repeated in adult male rats (250-300g) to assess if they could better tolerate the injury (n=6 with n=5 surviving, mortality rate of 17%). As mortality rates in males were much lower they were used for all further cervical contusion experiments. This study was also used to assess a variety of behavioural tests and identify which tests would be most useful in a larger treatment study.

Study two: Assessing the efficacy of LV-ChABC to treat a moderate cervical contusion injury in adult male rats (n=24; n=7 per group of contusion + LV-GFP and contusion + LV-ChABC, n=10 of contusion only).

Study three: Assessing the efficacy of LV-ChABC to treat a thoracic contusion injury in adult female rats (200-220g), using a thoracic contusion injury model of increased severity than that used in previous chapters (225 kdyne at T10; n=14; n=7 per group of contusion + LV-GFP and contusion + LV-ChABC).

4.2.2 Contusion injury surgery and treatment

Anaesthetized (ketamine, 60 mg/kg, and medetomidine, 0.25 mg/kg) adult rats were surgically prepared as previously described (section 2.2.2). A laminectomy was then performed at vertebral level C5 or T10 to expose the underlying spinal cord. Animals were then positioned and stabilised, with the impactor probe of an Infinite Horizon impactor (Precision Systems Instrumentation, Lexington, KY) positioned 2-4mm above the exposed spinal cord. The impactor probe then delivered an impact force of 225 kdyne to the spinal cord, inducing a moderate cervical (C5) contusion injury or a severe thoracic (T10) contusion injury. Following injury rats designated to a contusion only group had their wounds sutured and anaesthesia was then reversed using atipamezole hydrochloride (1mg/kg administered i.p.). All other animals received 0.5 μ l intraspinal injections of either LV-GFP or LV-ChABC immediately rostral and caudal to the contusion injury (one injection either side). The LVs used were the same as those used in chapter three (described in section 3.2.2), however the batch of LV-ChABC used in this chapter had a viral titer of 284 μ g/ml of P24 (compared to 479 μ g/ml in chapter 3). Injections were delivered at a rate of 200nl/min using an ultra micropump III (World Precisions Instrumentation, Europe). Following intraspinal injection wounds were closed and anaesthesia was reversed. Post-operative care was carried out as previously described (section 2.2.2).

4.2.3 Behavioural assessments

Animals were trained in all behavioural tasks for one week prior to surgery and were assessed on the day prior to surgery to provide baseline data for each task.

4.2.3.1 Forelimb locomotor scoring

A scoring system for assessing forelimb and hindlimb function following cervical SCI known as forelimb locomotor scoring (FLS) was used (Martinez et al., 2009). The

scoring system assesses forelimbs and hindlimbs separately, giving a maximum score of 20 for each. It involves assessing individual joint movements of all limbs, muscle tone of digits (flexed, atonic or extended), the presence of weight-support, paw positions, irregularity of movement, forelimb/hindlimb coordination and tail position. The various scoring parameters can be seen on the score sheet shown in figure 4.1. Scoring was carried out at post-injury days 2, 5 and 7 and weekly thereafter.

4.2.3.2 Horizontal ladder

The horizontal ladder task was performed and assessed as previously described (section 2.2.3.2) with the addition that animals receiving a cervical contusion injury had the total number of footslips made by their forelimbs assessed as well as their hindlimbs. Animals were assessed on the horizontal ladder once a week post-injury.

4.2.3.3 Inclined Plane

Animals were placed horizontally on a rubberised surface (to provide traction) initially held at an angle of 40° (Fig. 4.7D). If the rat could hold its position, with no signs of slipping, for 4 seconds then the incline was increased by 5°. This process was repeated until the incline reached an angle at which the rat could no longer stay stationary and began to slip. This was repeated twice more and an average angle of incline at which the rat began to slip was calculated from the three scores. Post-injury, animals were assessed once a week.

Rat:
Day post-op:
Date:
SCORE:
forelimb: (20)
hindlimb: (20)

Articular movements of the affected limbs						
	Forelimb			Hindlimb		
	Shoulder	Elbow	Wrist	Hip	Knee	Ankle
Absent	0	0	0	0	0	0
Slight	1	1	1	1	1	1
Normal	2	2	2	2	2	2

	Weight support	
	Stationary	Active
Forelimb	1	1
Hindlimb	1	1

	Stepping											
	Paw placement at initial contact					Paw orientation during lift off					Movement	
	Dorsal		Plantar			I		P				
	Flexed	Atonic	Extended			E			E	P	Regular	Irregular
Forelimb	0	1	2	0	1	1	2	1	1	2	2	1
Hindlimb	0	1	2	0	1	1	2	1	1	2	2	1

FL-HL coordination	
Absent	0
Occasional	1
Frequent	2
Consistent	3

Tail Position	
Up	1
Down	0

All the parameters have to be measured during the normal step and not during bot

Common part of the scale for the forelimb and hindlimb scores. These scores will be added up to the fore and hindlimb scores separately.

Figure 4.1. Forelimb locomotor score sheet. Scoring sheet taken from scoring system developed by Martinez *et al* (2009).

4.2.3.4 Forelimb grip-strength

Forelimb grip-strength was assessed once a week following injury using a forelimb grip-strength meter (Linton Instrumentation; Fig. 4.7B). This involved rats grasping onto a metal bar with their forelimbs, which was attached to a force transducer, and slowly being pulled backwards with gradually increasing force until the rat could no longer hold on and released the bar. The grip-strength meter gave a readout of the maximum force applied to the force transducer (i.e. the force at which the rat could no longer grip the bar) and this was recorded. An average was taken from three grip-strength readouts per session in weekly assessments.

4.2.3.5 Sticky tape test

Animals had a small (1x2cm) piece of electrical tape stuck to the plantar surface of their paw. They were then placed in a clear Perspex enclosure to which the rat had already been acclimatised. The enclosure was surrounded by mirrors to ensure the paw could be viewed from all angles. A stopwatch was started as soon as the rat was placed in the enclosure and the time noted when the first attempt to remove the tape was made (latency to sense) as well as the time then taken to completely remove the tape (latency to removal). This was repeated three times for each paw and an average latency to sense and latency to removal was calculated. This task was performed once a week post-injury.

4.2.3.6 Hanging test

In order to assess forelimb muscle fatigue and strength, animals were made to grasp an elevated horizontal bar with their forelimbs and then released so that they were hanging from the bar (Fig. 4.3F). A stopwatch was used to time how long each animal could

remain hanging on to the bar before it fatigued and/or lost its grip and fell. This was repeated three times and an average time was recorded for each session. This task was performed once a week following injury.

4.2.3.7 Digigait

Animals placed on a Digigait (Mouse Specifics Inc.) treadmill set at a speed of 25 cm/s at various post-injury time-points (2, 4, 6, 7 and 8 weeks post-injury). The treadmill belt was transparent and a camera placed underneath the treadmill allowed the ventral surface of the rat to be recorded whilst running (Fig. 4.4A). This video was then analysed using the associated Digigait software which recognised the red pixels of the paws in the video and used this information to digitally paint each paw (Fig. 4.4B). A wide variety of gait parameters could then be calculated by the Digigait software including stride length and duration (for hindlimbs and forelimbs), paw area at peak stance (the maximum area of the paw in contact with the treadmill during the stance phase of locomotion) and gait symmetry (the ratio of hindlimb/forelimb stride length, providing a correlate of forelimb/hindlimb coordination).

4.2.4 Electrophysiological assessment of function

4.2.4.1 Conduction in long-distance, ascending sensory fibres travelling in the dorsal columns

Conduction of sensory fibres through the lesion site was assessed as previously described in chapters two and three (sections 2.2.5.1 and 3.2.4.1). In order to assess a cervical contusion this was slightly adapted such that a lumbar laminectomy was first performed to expose usable lengths of dorsal roots to record from (L3-S1), followed by a separate laminectomy at cervical level to expose the injury site and allow access for stimulating electrodes rostral and caudal to the injury (Fig. 4.8A). In order to assess a

severe thoracic contusion this protocol was again adapted, such that whole dorsal roots were recorded from rather than teased dorsal root filaments (L3-S1 were still assessed; Fig. 4.12A), this was due to the reduced probability of finding unitary activity when stimulating rostral to the lesion. Recording from whole dorsal roots allowed all remaining functional sensory fibres that extended from the root to beyond the lesion site to be recorded from at once, giving a sensory compound action potential (SCAP). SCAPs were averaged from 32 traces for each dorsal root using LabChart software (AD instruments) and measurements of peak-to-peak amplitude as well as latency to peak response were determined for each averaged SCAP (Fig. 4.12B). For each dorsal root the averaged SCAP elicited when stimulating rostral to the lesion was converted into a percentage of the average SCAP elicited when stimulating caudal to the lesion, allowing a comparison of conduction through the injury site to conduction of the intact portion of the dorsal column pathway. SCAPs were always recorded following supramaximal stimulation (800 μ A).

4.2.4.2 Corticospinal tract function

For the duration of each electrophysiological experiment animals were deeply anaesthetised with urethane (1.25g/kg; administered i.p.) and depth of anaesthesia was regularly assessed by monitoring pedal withdrawal reflexes and respiratory rate. Core temperature was maintained close to 37°C using a self-regulating heated blanket. A cervical laminectomy was performed to expose the spinal cord at the injury site and surrounding area and a craniotomy was performed to reveal the sensory motor cortex on the right hand side of the brain. A concentric bipolar electrode was lowered to a depth of 1mm within the cortex and a silver ball recording electrode was placed medially on the contralateral spinal cord surface rostral to the lesion (Fig. 4.9A). Post-synaptic potentials were then recorded following cortical stimulation (4 square-wave pulses at

500 Hz, 200 μ A, 200 μ s, delivered every 2 seconds), the stimulating electrode was then repositioned to find the optimal stimulation site (generally 2mm lateral and 1mm rostral to Bregma) where it remained for the duration of the experiment. 64 responses were then averaged using LabChart software (AD instruments), first whilst recording immediately rostral to the lesion site and then whilst recording immediately caudal to the lesion (Fig. 4.9B). The amplitude of the averaged response recorded caudal to the lesion was then converted into a percentage of the amplitude of the averaged response recorded rostral to the lesion.

4.2.5 Histological assessment of spinal atrophy, tissue damage and cavitation

Tissue preparation and eriochrome cyanine histochemistry were performed as previously described (section 2.2.6.1 and 2.2.6.3). Sections were examined using a Zeiss Axioskop microscope and pictures were taken of sections at 800 μ m intervals throughout the lesion site using a Zeiss AxioCam MRm. Images were analysed using Axiovision software, which allowed the tracing of the spinal cord perimeter, white matter, damaged tissue, and cavity for each image captured, giving the total area for these measurements in each section. Measurements of tissue damage incorporated both the cavity and any remaining tissue debris, whilst cavity measurements were made only around areas completely cleared of tissue debris. The lesion epicentre was defined as the section from each animal with the largest cavity area and quantification was carried out at 800 μ m intervals from 2.4mm caudal to 2.4mm rostral to the epicentre. White matter, cavity area and tissue damage were calculated as a percentage of spinal cord area (i.e. area within the tracing of spinal cord perimeter) for each section.

4.3 Results

4.3.1 Study one: Assessment of behavioural and anatomical deficits following cervical contusion injury

4.3.1.1 *Locomotor function*

In order to assess gross locomotor function in both male and female rats a forelimb locomotor scale (FLS) was used (Martinez et al., 2009). Prior to receiving a 225 kdyne contusion injury at C5 all animals achieved the maximum score of 20 for both forelimb (Fig. 4.2A) and hindlimb (Fig. 4.2B) function, representing fully coordinated, weight-supported steps with no abnormalities (see Fig. 4.1 for FLS score sheet). Uninjured animals (n=5 female rats) maintained forelimb and hindlimb scores of 20 throughout the 8 week study period. Following cervical contusion, both the male and the female cohort of rats showed a very similar initial deficit and subsequent pattern of spontaneous recovery. At 2 days post-injury both cohorts had a mean FLS score of 2 for forelimbs, whilst mean hindlimb scores were 3 ± 0.89 and 4 ± 1.2 for the female and male cohorts respectively. These scores indicated that at an early post-injury stage there were only slight movements in one or two joints for each limb and all paws were flexed. All animals gradually recovered to a mean score of ~15 for hindlimbs and forelimbs, generally taking around 4-5 weeks to reach their maximum score (Fig. 4.2A+B). These scores reflected an ability to take weight-supported steps with all limbs as well as only slight abnormalities with paw placement and digit position. Movement remained irregular in all animals and no animal achieved consistent forelimb-hindlimb coordination. Whilst recovery from a score of 2 at 2 days post-injury to a score of 15 at later time points represents a significant recovery of gross locomotor function ($p < 0.01$, one-way ANOVA, Tukey's *post-hoc*), all animals remained obviously and significantly

impaired compared to uninjured controls ($p < 0.001$, two-way RM ANOVA, Bonferroni *post-hoc*).

Skilled locomotor function was assessed using the horizontal ladder task, with animals receiving a score for hindlimb and forelimb slips separately. As with FLS scores, ladder scores were initially severely impaired (males - 57 ± 5 and 29.4 ± 2.2 ; females - 44 ± 10.3 and 36 ± 7.1 for forelimbs and hindlimbs, respectively), but displayed a level of spontaneous recovery (Fig. 4.2C+D). Interestingly, ladder scores appeared to plateau slightly earlier than FLS scores, with most animals achieving their maximum scores by 3 weeks post-injury (males - 39.4 ± 1.8 and 22.6 ± 2.3 ; females - 30 ± 3.5 and 16 ± 2 for forelimbs and hindlimbs respectively). The male cohort appeared to have a more significant forelimb impairment than females when assessed using the ladder task, although this was only significantly different at 2 weeks post-injury ($p < 0.001$, two-way RM ANOVA, Bonferroni *post-hoc*). Whilst performance on the ladder task plateaued at a slightly earlier time point compared to FLS scores, during behavioural assessment impairments were most obvious whilst performing the ladder task. Functional deficits remained significant compared to uninjured controls throughout the study period (8 weeks; $p < 0.001$, two-way RM ANOVA, Bonferroni *post-hoc*).

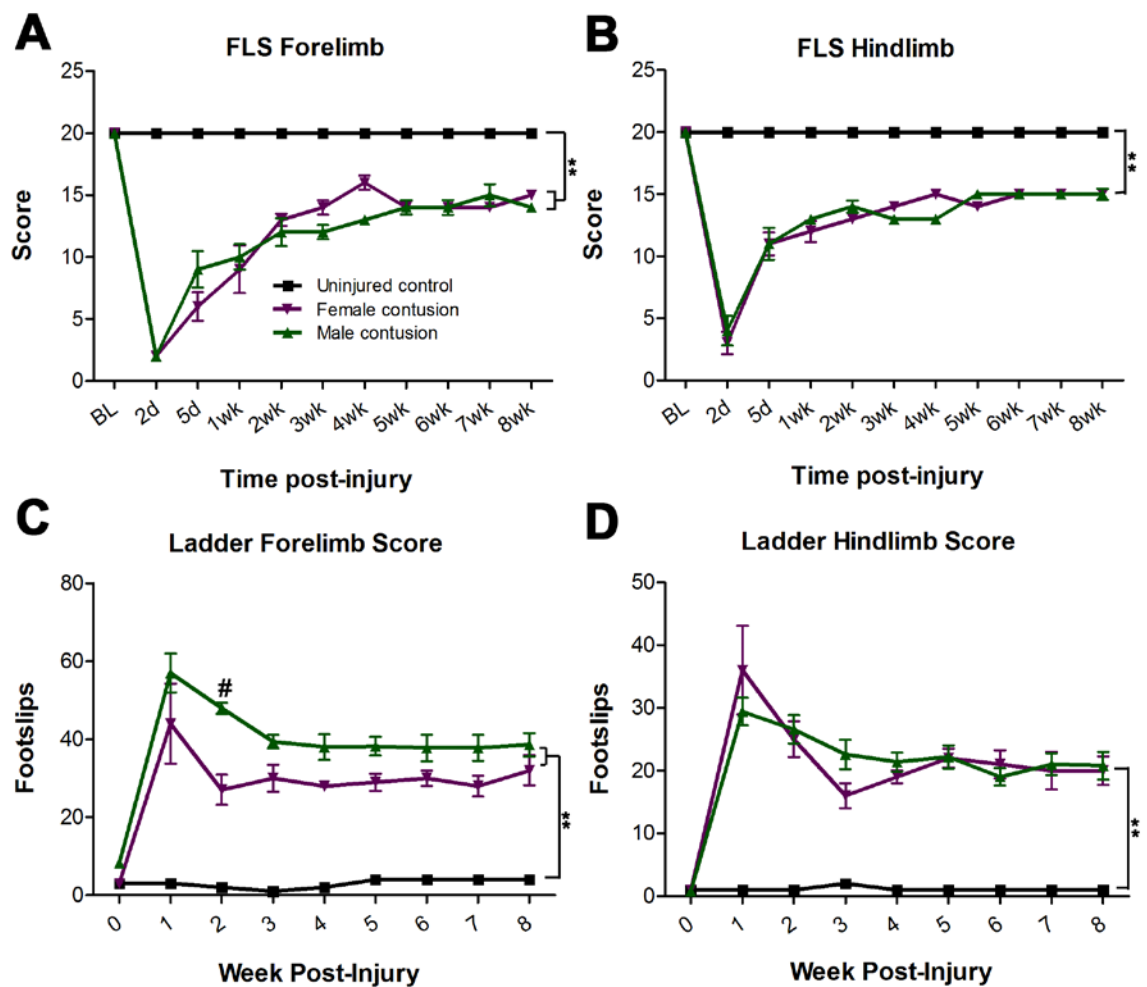


Figure 4.2. Cervical contusion results in significant forelimb and hindlimb locomotor deficits. Behavioural scoring using the forelimb locomotor scale (FLS) indicates a severe deficit in both forelimb (A) and hindlimb (B) function at acute time-points following a 225 kdyne C5 contusion injury, followed by gradual, spontaneous improvements in both the male and female cohort. (C,D) Quantification of total footslips whilst performing the horizontal ladder task indicates that skilled locomotor function follows a similar pattern of functional impairment and spontaneous recovery. Injured males make more forelimb footslips than injured female rats and this is significant at 2 weeks post-injury. ** indicates a significant difference ($p < 0.01$) between uninjured animals and both injured cohorts, # indicates a significant difference (< 0.05) between the injured male and female cohorts, two-way RM ANOVA, Bonferroni *post-hoc*. $n = 5$ per group.

4.3.1.2 Forelimb behavioural tasks

In addition to the assessment of locomotor function using tests similar to those used in previous chapters to assess functional recovery following a thoracic contusion injury, additional behavioural tasks were utilised in order to assess varying aspects of injury and recovery, particularly focussing on forelimb function (Fig. 4.3). The inclined plane assessed proprioceptive function, examining ability to use all four limbs to balance and remain stationary at a gradually increasing angle of inclination. Both the male and female cohort of animals followed the same pattern of recovery using this test, unable to balance at any angle $>50^{\circ}$ at 1 week post-injury but recovering to a mean score of $58^{\circ} \pm 1.8$ by 4 weeks post-injury (Fig. 4.3A). Uninjured animals were able to balance at all angles $\leq 65^{\circ}$ throughout the study, indicating that, whilst this test detected a significant difference between injured and uninjured animals ($p < 0.01$, two-way RM ANOVA, Bonferroni *post-hoc*), there may be little scope for further improvements using this test to assess a potential therapeutic. Assessment of forelimb grip-strength detected a dramatic and highly significant loss of function following cervical contusion (Fig. 4.3B; $p < 0.001$ compared to uninjured controls, two-way RM ANOVA, Bonferroni *post-hoc*). Animals were capable of exerting forces of $\sim 700\text{g}$ prior to injury, which was reduced to $252\text{g} \pm 32$ and $321\text{g} \pm 50$ for males and females respectively by 1 week post-injury and failed to display any notable recovery beyond this. The attachment of a small piece of sticky tape to the plantar surface of the forepaw allowed an assessment of forelimb sensory (time taken to notice the tape) and motor function (time taken to remove tape once it was noticed) (Fig. 4.3C+D). Only the male cohort of animals performed this task (the task was added in once the female cohort of animals had almost completed the behavioural study) and, whilst there were notable increases in the time

taken to sense and remove tape at all post-injury time-points, none of the post-injury data was significantly different to that achieved at baseline. This was due to the high levels of variability inherent in this data and the relatively small group sizes. As well as there being a wide range in the ability of animals to perform this task, some animals often appeared to lose motivation to remove tape and this further confounded results. Finally, animals were also assessed using a hanging test (Fig. 4.3E). This involved animals grasping onto an elevated horizontal bar with their forelimbs and timing how long they could suspend themselves before fatiguing and letting go (pictured in Fig. 4.3F). The male cohort of animals would not cooperate in performing this task, releasing their grip on the horizontal bar almost immediately after being placed on it. Results obtained from uninjured and injured female rats were highly variable and displayed no obvious pattern from week to week, including no post-injury effect (Fig. 4.3E).

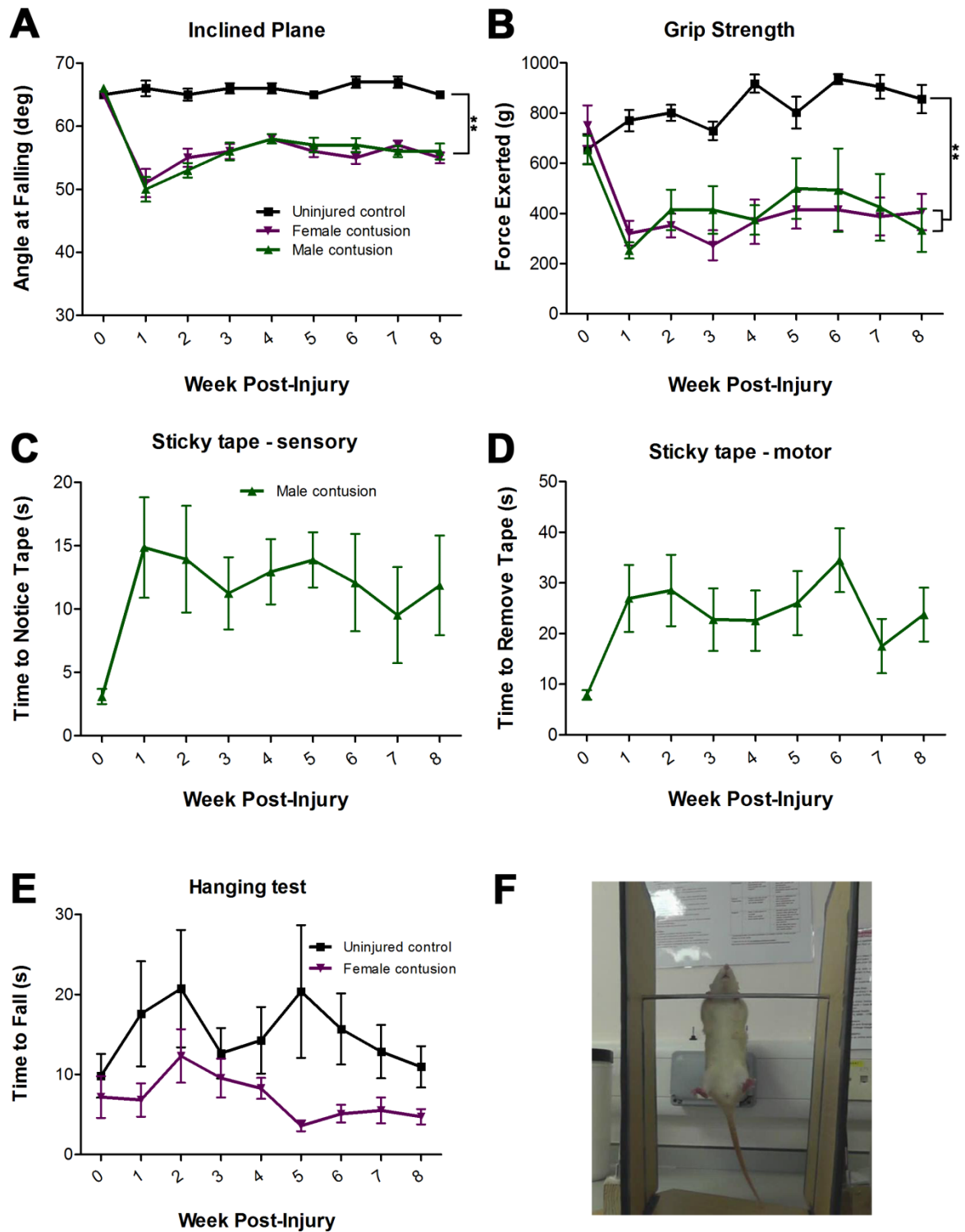


Figure 4.3. Cervical contusion causes deficits in a variety of behavioural functions. (A,B) Both the male and female cohorts of injured rats show significant deficits in proprioceptive function, as shown by performance on the inclined plane test (A), and forelimb grip-strength, as indicated by maximum force readouts from a grip-strength meter (B). Performance in both the

sensory (C) and the motor (B) component of a sticky tape removal task declines following injury in male rats, however results are very variable and this decline is not significant. (E) Female rats performing a forelimb hanging test display no significant deficit post-injury. (F) Photograph of rat performing the hanging test. ** indicates $p < 0.01$ two-way ANOVA, Bonferroni *post-hoc*. $n = 5$ per group.

4.3.1.3 Digigait gait analysis

A wide variety of gait parameters were assessed using the Digigait (Mouse Specifics, Inc.) treadmill and gait analysis software system (Fig. 4.4). The system records animals running at a set speed (25 cm/s in this study) through a transparent treadmill (i.e. from a ventral perspective, Fig. 4.4A) and the software then digitally paint the paws by recognising red pixels (Fig. 4.4B). The software can then analyse a large number of gait parameters, a number of which were examined in this study and a selection of parameters in which there was a notable difference in injured and uninjured animals are presented in Figure 4.4C-H. The majority of animals in the male cohort of the study refused to run on the treadmill and therefore data is only presented for uninjured and injured female animals. Data obtained using Digigait suggested that deficits in forelimb gait parameters were particularly apparent, as highlighted by the prominent deficits in forelimb stride duration (Fig. 4.4C) and length (Fig. 4.4F) that persisted for the duration of the study. Hindlimb gait parameters, such as stride duration (Fig. 4.4D) and length (Fig. 4.4G), were initially significantly impaired at early post-injury time points, but recovered to values similar to those seen in uninjured animals by the sub-acute and chronic post-injury phase. Interestingly, the maximum area of the hindpaw recognised by the Digigait software was significantly less in injured animals compared to their uninjured counterparts, particularly at chronic post-injury time points (Fig. 4.4E). This

suggests that injured animals were not placing the full plantar surface of their hindpaws onto the treadmill during stepping. Gait symmetry is a measure of hindlimb/forelimb stride length ratio, indicating the extent of coordination achieved. This parameter showed a particularly notable effect of injury (Fig. 4.4H), substantiating the observation that no animal achieved consistent coordination during FLS scoring sessions.

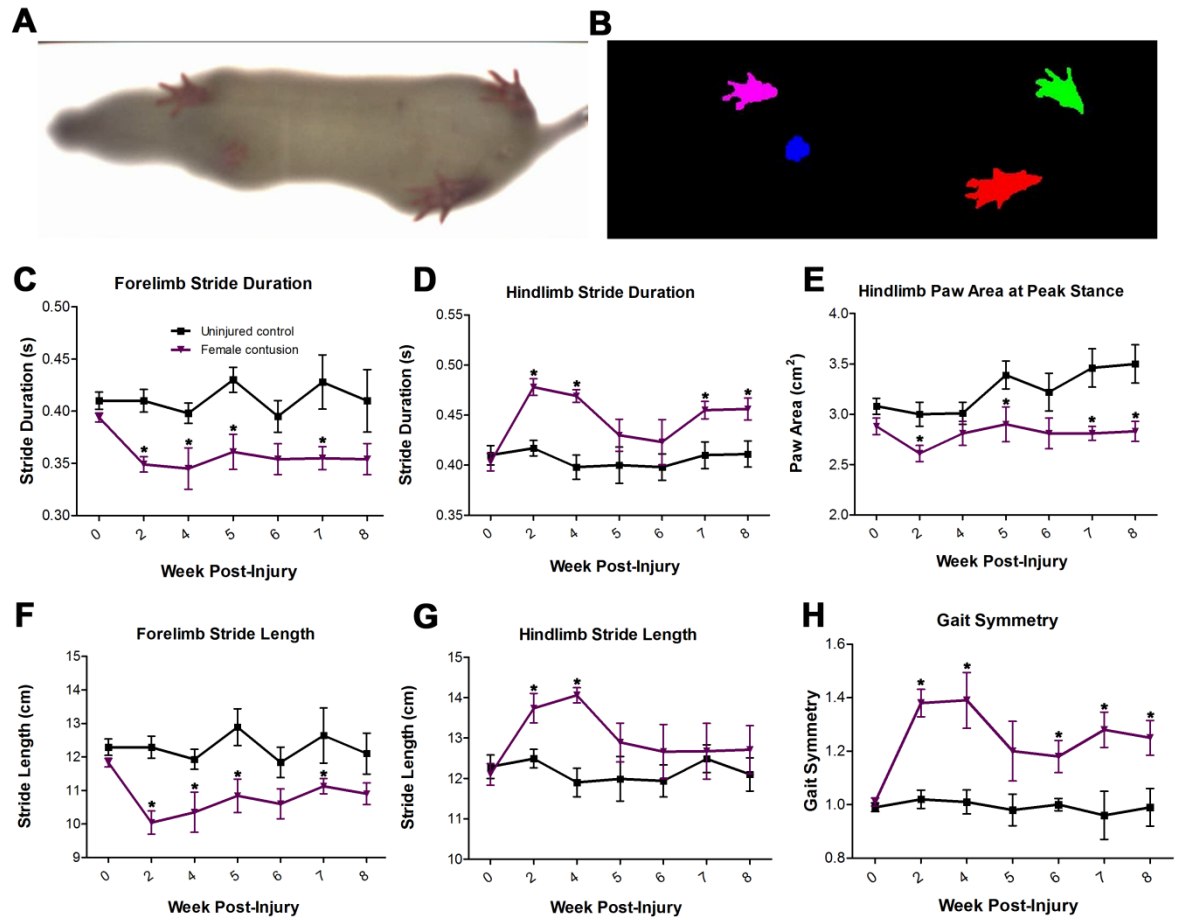


Figure 4.4. A variety of gait parameters are affected by cervical contusion injury. (A) Still image taken from video used for Digigait analysis, Digigait software digitally paints the paws (B) by recognising red pixels in order to perform automated gait analysis. Gait parameters that are significantly affected by a cervical contusion injury include decreased forelimb stride duration (C), increased hindlimb stride duration (D), decreased hindlimb paw area at peak stance (E), decreased forelimb stride length (F), increased hindlimb stride length (G) and a lack of gait symmetry (H). * indicates a significant difference from uninjured controls, two-way RM ANOVA, Bonferroni *post-hoc*. n=5 per group.

4.3.1.4 Quantification of tissue damage following cervical contusion

Erichrome cyanine staining of serial sections through the injured area of the spinal cord was used in order to quantify the spread of cavitation and tissue damage (Fig. 4.5). At injury epicentre the mean cavity area was $27.3\% \pm 2.6$ of the total spinal cord section, whilst $50.5\% \pm 2.9$ of the total spinal cord area consisted of damaged tissue (cleared cavity plus any remaining tissue debris) (Fig. 4.5B+C). The tissue damage caused by cervical contusion did not appear to spread beyond 1.6mm rostral or caudal to the lesion epicentre, as indicated by quantification of tissue damage and cavitation (Fig. 4.5B+C).

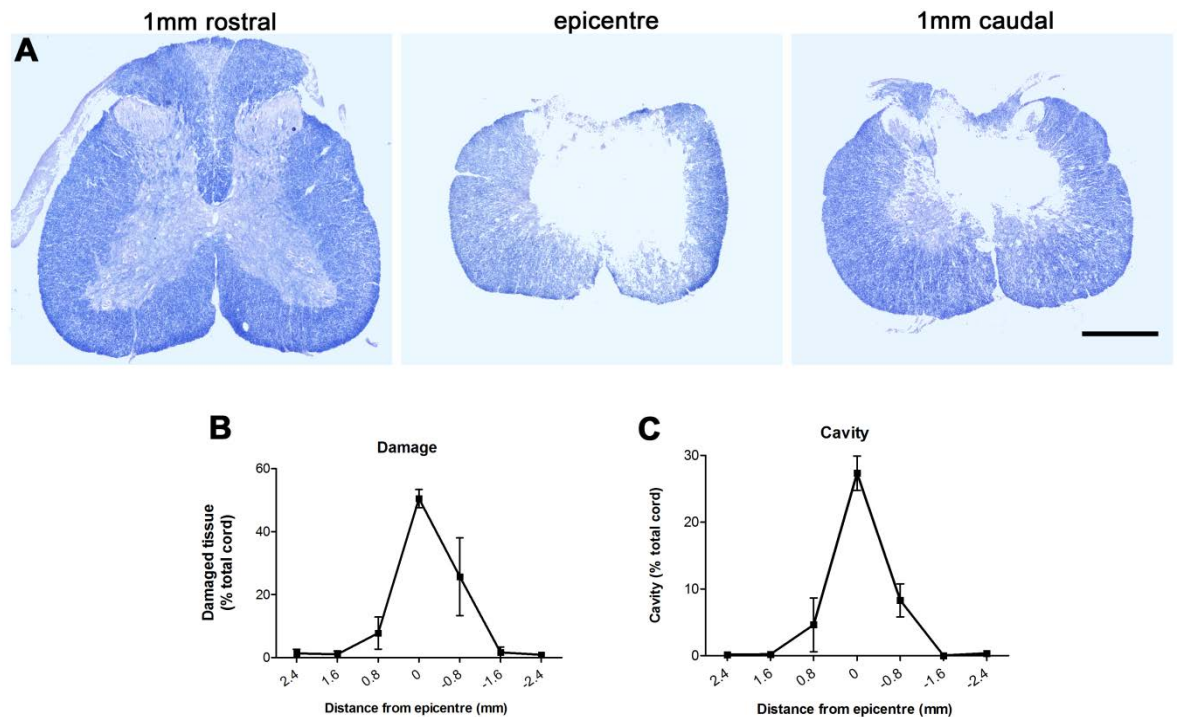


Figure 4.5. Cervical contusion causes tissue damage and cavitation. (A) Representative images of transverse sections of injured spinal cord at the lesion epicentre and 1mm rostral and caudal of this. Sections are stained using eriochrome cyanine to differentiate white and grey matter. (B,C) Quantification of tissue damage and cavitation reveals that $50.5\% \pm 2.9$ of spinal cord area consists of tissue debris and cavity at lesion epicentre (B), whilst the cavity itself takes up $27.3\% \pm 2.6$ of the spinal cord at lesion epicentre (C). Tissue damage and cavitation do not spread beyond 1.6mm rostral or caudal to the injury epicentre. Scale bar=1mm, n=4 per group.

4.3.2 Study two: Assessment of LV-ChABC using a cervical contusion injury model

4.3.2.1 Behavioural assessment of function

Due to decreased mortality rates in the male cohort of rats whilst optimising the cervical contusion injury model, male rats were subsequently used in order to assess the effects of LV-ChABC following a cervical contusion injury. The behavioural tests evaluated during the optimisation of the model which displayed low levels of variability, a significant post-injury deficit and provided a reasonable window for therapeutic improvement were selected as outcome measures in this assessment. The tests selected were the FLS, the horizontal ladder, forelimb grip-strength and inclined plane.

Following treatment of a 225 kdyne C5 contusion with LV-ChABC no improvements in gross locomotor function were observed when assessed using the FLS (Fig. 4.6A+B) However, significant functional improvements in skilled forelimb and hindlimb locomotion were detected when assessed using the horizontal ladder (Fig. 4.6C+D). Whilst all animals were similarly impaired at acute post-injury time-points (2 days to 1 week post injury), animals treated with LV-ChABC displayed significantly improved functional recovery on the horizontal ladder compared to all other treatment groups at all post-injury time points beyond 3 weeks ($p < 0.01$; two-way RM ANOVA, Bonferroni *post-hoc*), such that by 10 weeks post-injury LV-ChABC treated animals were making 33.3 ± 2.2 forelimb and 19.6 ± 2.1 hindlimb slips compared to 54.1 ± 6.1 and 29.4 ± 1.2 for contusion only and 61.7 ± 3 and 30.3 ± 2.8 for contusion + LV-GFP. LV-ChABC treated animals also displayed significant improvements in forelimb grip-strength compared to all other treatment groups (Fig. 4.7A+B; $p < 0.05$, two-way RM ANOVA). There were no detectable differences between groups when comparing

proprioceptive function using the inclined plane task (Fig. 4.7C+D). These results indicate that LV-ChABC leads to significant functional improvements following a cervical contusion injury and that these improvements are most prominent in the horizontal ladder task.

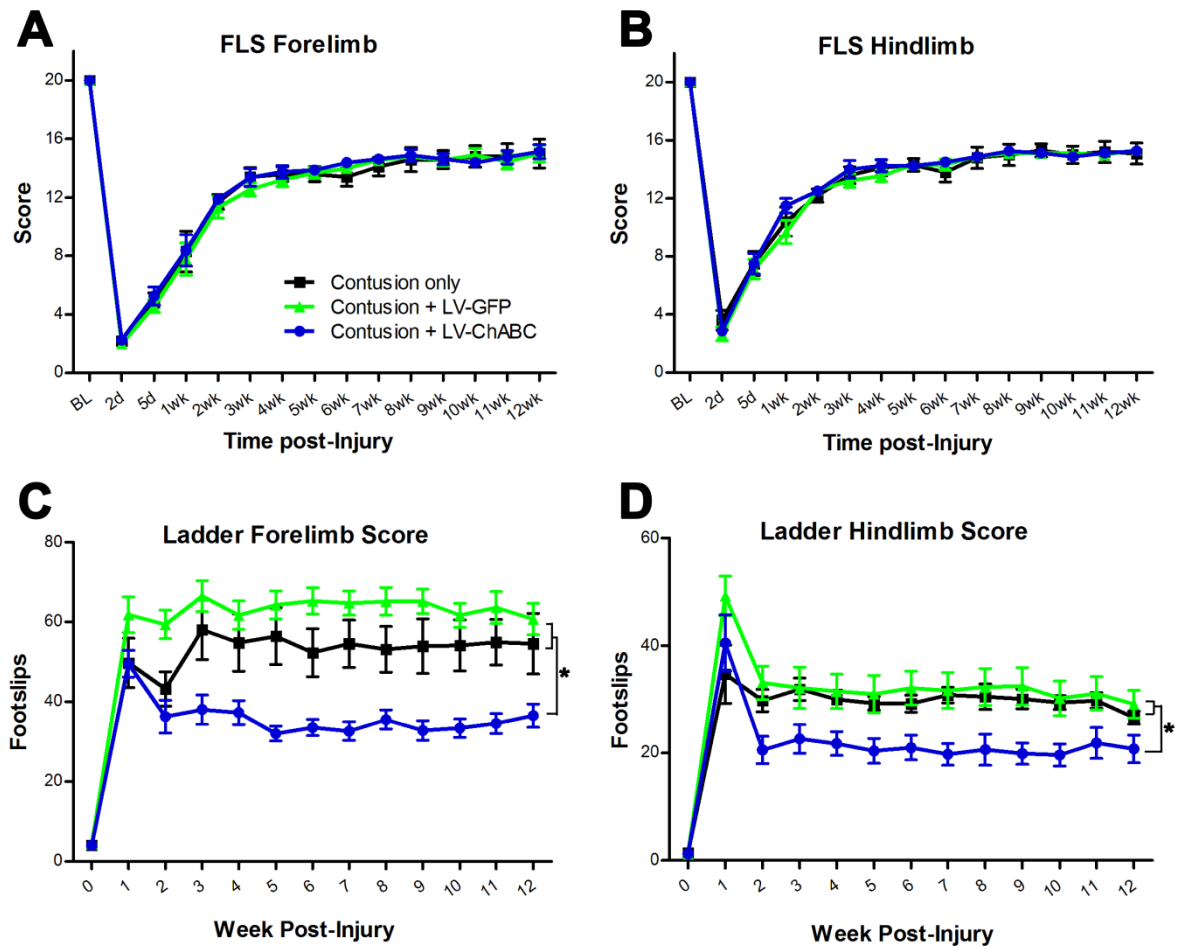


Figure 4.6. LV-ChABC treatment significantly improves skilled locomotion following cervical contusion injury. (A,B) LV-ChABC has no effect on gross locomotor function of forelimbs (A) or hindlimbs (B), as assessed using the FLS. (C,D) A significant decrease in total hindlimb (C) and forelimb (D) footslips shows that treatment of a cervical contusion injury with LV-ChABC leads to significant improvements in skilled locomotor function (* $p < 0.05$, two-way RM ANOVA, Bonferroni *post-hoc*, $n = 7$ per group).

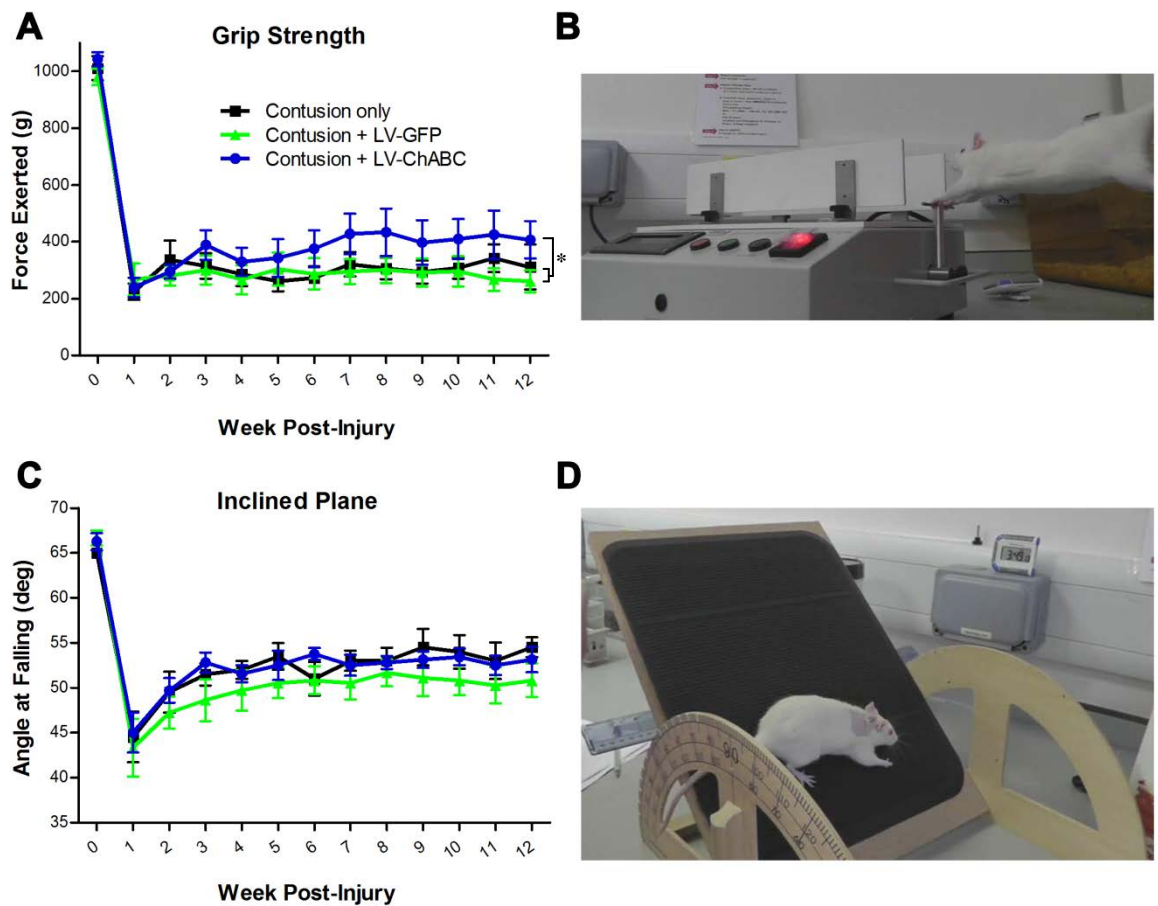


Figure 4.7. Treatment with LV-ChABC leads to modest improvements in forelimb grip-strength. (A) Maximum force readouts from a grip-strength meter indicate that treatment with LV-ChABC leads to significant improvements in forelimb grip-strength ($p < 0.05$, two-way RM ANOVA). (B) Photograph of rat grasping the bars of a grip-strength meter, the rat is gradually pulled backwards until it can no longer hold onto the bar and the device displays the maximum force exerted. (C) Behavioural results from the inclined plane test show that rats from all treatment groups lose balance between ~ 50 - 55° , indicating that treatment has no effect on proprioceptive function. (D) Photograph of inclined plane apparatus. $n=7$ per group.

4.3.2.2 Electrophysiological assessment of sensory and corticospinal tract function

Electrophysiological experiments were next performed in order to assess the extent of long distance, sensory fibre conduction across the cervical contusion site (technique used in chapters two and three; schematic representation Fig. 4.8A). The antidromic activation of sensory dorsal column fibres caudal to the injury site allowed the recording of unitary activity from lumbar dorsal roots. Conduction through the injury site could then be assessed by stimulating rostral to the lesion and determining the percentage of single units still present in each dorsal root filament. LV-ChABC treated animals displayed significantly improved levels of conduction through the injury site in comparison to all other treatment groups ($16.7\% \pm 1.6$ compared to $4.7\% \pm 1.6$ and $4.3\% \pm 1.8$ for contusion only and contusion + LV-GFP; $p < 0.01$, one-way ANOVA, Tukey's *post-hoc*; Fig 4.8B).

In addition to the electrophysiological assessment of an ascending sensory pathway, electrophysiology was also used to assess the function of a descending, motor pathway; the CST. The CST was discretely activated using a tungsten, concentric bipolar electrode lowered into the appropriate area of the motor cortex, extracellular recordings were then made from rostral and caudal to the injury site in order to assess CST functionality through the lesion (Fig. 4.9A). Amplitude analysis of CST potentials recorded rostral and caudal to the lesion site revealed that LV-ChABC treated animals had the largest CST response caudal to the lesion in comparison to the response elicited rostral to the lesion following stimulation at the same site (Fig. 4.9). Whilst the mean CST response recorded below the lesion in LV-ChABC animals was double that observed in any other group ($25.51\% \pm 18$ compared to $13.4\% \pm 8$ and $1.3\% \pm 0.7$ for

contusion only and contusion + LV-GFP respectively), this was not significant due to high variability in CST responses and small group sizes (n=3 per group) (Fig. 4.9E).

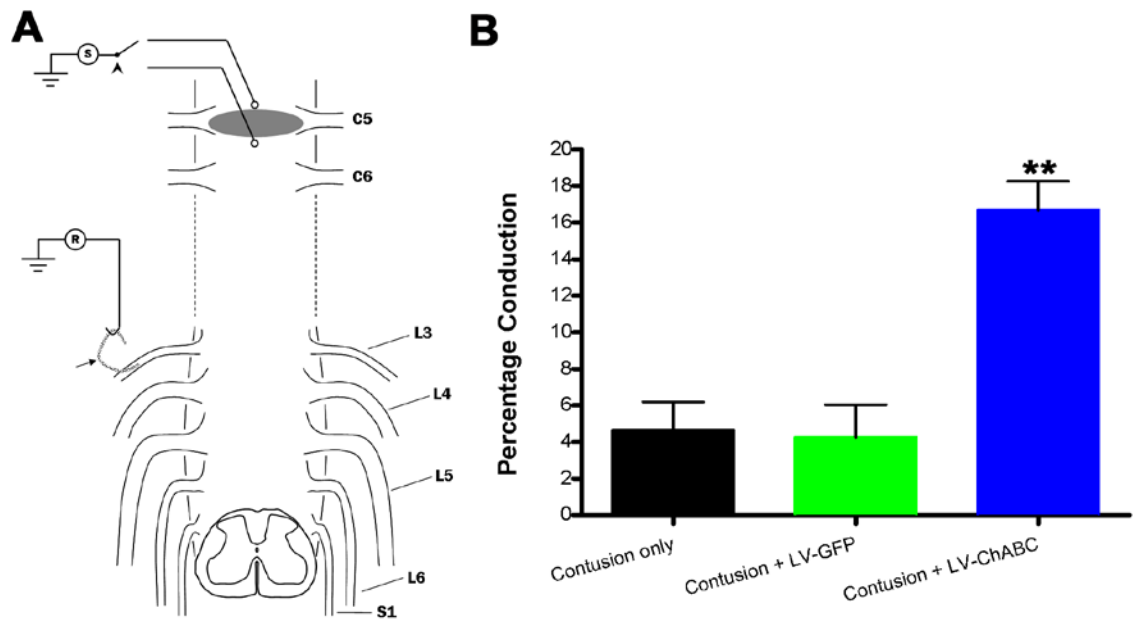


Figure 4.8. Sensory conduction through the injury site is dramatically improved in LV-ChABC treated animals. (A) Schematic representation of electrophysiological protocol used to assess conduction of ascending sensory fibres through a cervical contusion site. At 12 weeks post-injury the dorsal columns were first stimulated (S) caudal to the lesion site whilst recording (R) antidromic unitary activity from teased dorsal root filaments, the percentage of single units that could still be elicited when stimulation was switched rostral to the lesion site was quantified. (B) Quantification of sensory fibre conduction through the cervical contusion site shows that LV-ChABC treatment leads to a significant increase in functional sensory fibres through the lesion (16.7%±1.7 compared to 4.7%±1.6 and 4.3%±1.8 for contusion only and contusion + LV-GFP respectively, n=4 per group). **p<0.01, two-way RM ANOVA, Bonferroni *post-hoc*.

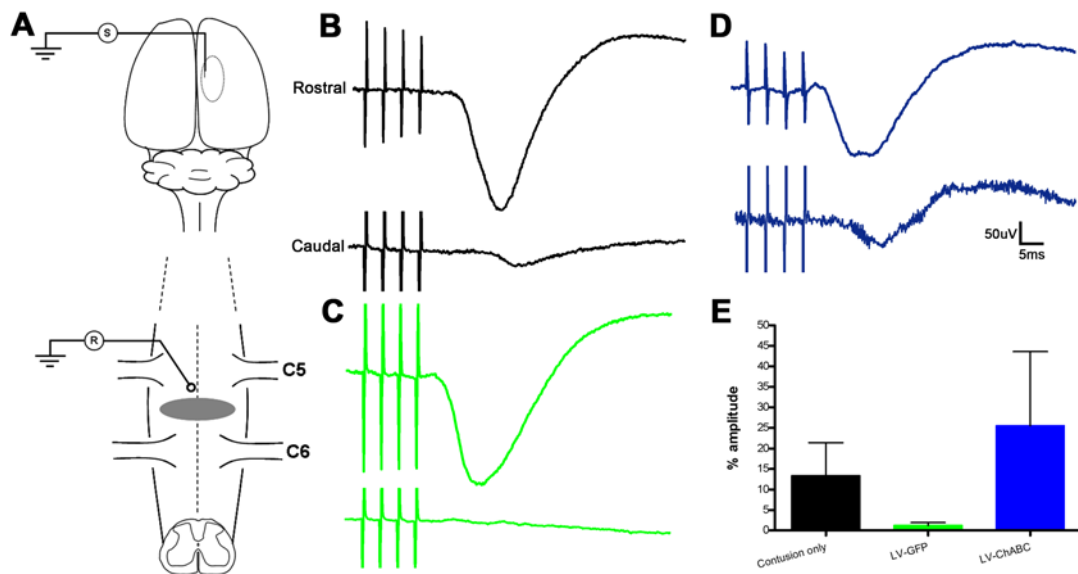


Figure 4.9. Cortical CST activation elicits the largest below lesion response in LV-ChABC treated animals. (A) Schematic representation of electrophysiological protocol used to assess CST function. At 12 weeks post-injury the CST was activated at the level of the cortex following a train of four high frequency pulses using a concentric, bi-polar electrode (S), extracellular CST potentials were recorded rostral and caudal to the cervical contusion via a silver ball electrode (R). (B-D) Example traces recorded rostral and caudal to the lesion site in contusion only (B), contusion + LV-GFP (C) and contusion + LV-ChABC (D) animals. (E) Quantification of the amplitude of potentials recorded from caudal to the lesion site as a percentage of the amplitude of potentials recorded from rostral to the lesion site whilst stimulating at the same cortical site. LV-ChABC treated animals had the largest amplitude responses caudal to the lesion site, however this was not significant. n=3 per group.

4.3.2.3 Assessment of cavitation

Erichrome cyanine staining was performed on histological tissue sections in order to quantify cavity formation at lesion epicentre and its rostrocaudal spread (Fig. 4.10). At lesion epicentre the mean cavity size of LV-ChABC treated animals (Fig. 4.10C) was notably reduced in comparison with contusion only (Fig. 4.10A) and LV-GFP treated animals (Fig. 4.10B) ($14.3\% \pm 4.8$ compared to $22.3\% \pm 2.6$ and $19.9\% \pm 6.6$ for contusion only and LV-GFP respectively; Fig. 4.10D), although this effect did not reach significance ($p > 0.05$ two-way RM ANOVA). In addition, there was also less rostrocaudal spread of cavity formation in LV-ChABC treated animals; there was minimal cavity present in the tissue sections analysed immediately rostral or caudal to the epicentre (0.8mm from epicentre) in LV-ChABC treated animals, whilst in contusion only and LV-GFP treated animals cavities were still approximately 10% of the total cord area in these tissue sections (Fig. 4.10D).

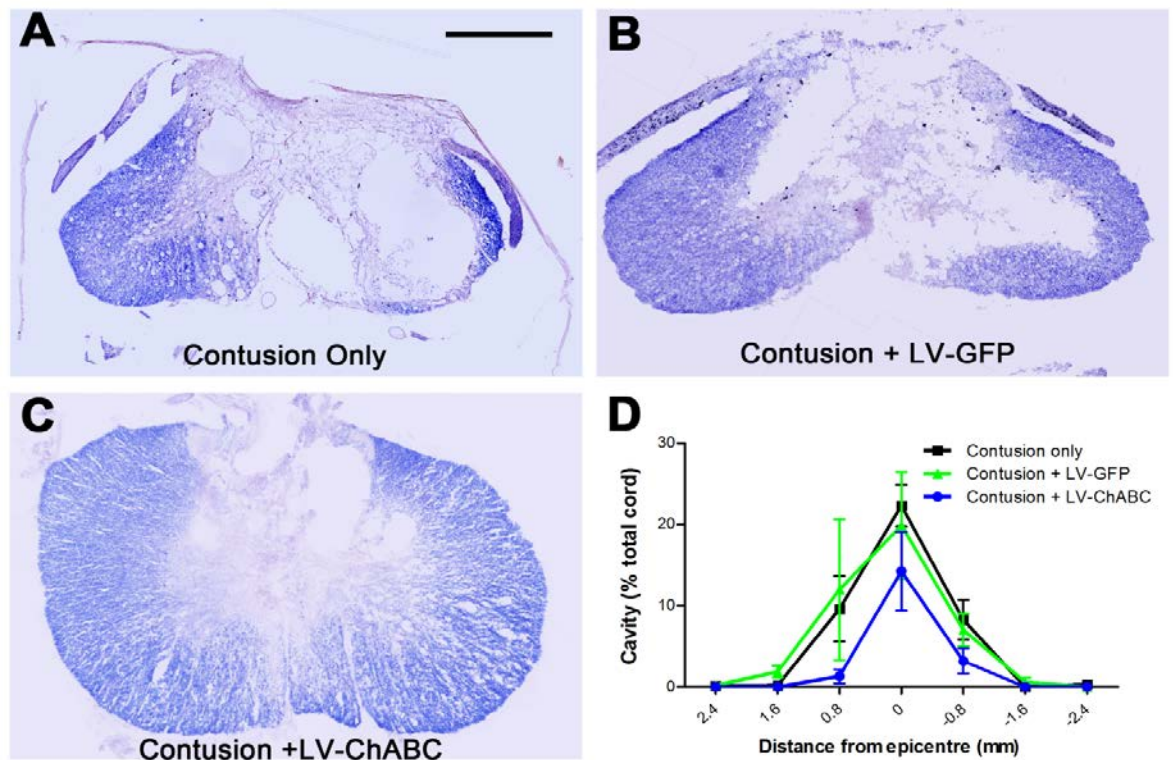


Figure 4.10. LV-ChABC reduces cavity formation at a cervical contusion injury site. Representative images of erichrome cyanine stained tissue sections at lesion epicentre taken from contusion only (A), LV-GFP (B) and LV-ChABC (C) treated animals at 12 weeks post-injury. (D) Quantification of cavity formation and its rostro-caudal spread in relation to lesion epicentre. Cavitation in LV-ChABC treated animals appears to occur to a lesser extent in LV-ChABC treated animals in comparison to contusion only and LV-GFP treated animals. This is the case at lesion epicentre and in the rostro-caudal spread of the cavity. Scale bar = 1mm, n=4 per group.

4.3.3 Study three: Assessment of LV-ChABC efficacy in a severe thoracic contusion injury model

In order to address the important issue of assessing the efficacy of a potential therapeutic in a variety of clinically relevant injury models, the effects of LV-ChABC were also assessed using a more severe thoracic contusion injury than that used in chapters two and three. The severity of this injury meant that animals did not recover sufficient hindlimb function to perform the horizontal ladder task and, therefore, behavioural recovery was assessed using the BBB locomotor rating scale only. As had been previously observed in both thoracic and cervical contusion injuries, LV-ChABC failed to promote significant improvements in gross locomotor function when assessed using BBB or FLS scoring systems (Fig. 4.11). At 2 days post-injury all animals were severely impaired, very rarely displaying movement of any kind in any hindlimb joints, as indicated by the mean scores of 0.2 in both treatment groups. Recovery in this more severe injury was slower than that observed following a moderate thoracic contusion injury, taking 3 weeks to plateau (rather than the 2 weeks generally observed following moderate injury). Both groups of animals recovered to an identical BBB score of 9.9 ± 0.5 at this 3 week time point and displayed no further recovery by the end of the study period the following week (Fig. 4.11).

As in previous chapters, conduction of long distance, ascending sensory fibres was assessed using electrophysiology (Fig. 4.12). Due to the severity of this injury, single units of activity were rarely recorded when stimulating rostral to the lesion and recording from teased dorsal root filaments, antidromically activated sensory compound action potentials (SCAPs) were therefore recorded from whole dorsal roots instead (Fig. 4.12A). These SCAPs were typically composed of a large early negative component

followed by a slightly smaller and later positive component and measurements of peak-to-peak amplitude as well as the latency to the peak of the initial negative component were made (Fig. 4.12B). Sensory conduction through the lesion site of LV-ChABC treated animals was more than double that observed in LV-GFP treated animals ($12.3\% \pm 3$ compared to $5.5\% \pm 1.6$); whilst this difference did not reach significance this is likely due to insufficient animals numbers ($n=4$ per group; $p=0.07$, unpaired t-test). Whilst the slightly shorter latency to peak response observed in LV-ChABC treated animals suggests that conduction may be slightly faster through the lesion than in LV-GFP treated controls, this effect was also not statistically significant ($p>0.05$, unpaired t-test).

In addition to functional assessments, the effects of LV-ChABC using this injury model were also assessed through evaluation of tissue sparing through the injury site (Fig. 4.13). As spinal tissue for histological processing was harvested at 4 weeks post-injury in this study, there was minimal cavitation apparent following staining of tissue sections with eriochrome cyanine. Instead damaged tissue and tissue debris were apparent throughout the lesion site (Fig. 4.13A+B). As cavity size could not be measured, the extent of white matter sparing was instead quantified. There was a significantly higher proportion of spared white matter in LV-ChABC treated animals in comparison to those treated with LV-GFP ($p<0.001$, two-way RM ANOVA), this was apparent at injury epicentre ($26.1\% \pm 2$ vs $12.4\% \pm 2.9$; $p<0.05$, Bonferroni *post-hoc*) as well as in the rostro-caudal spread of tissue damage (Fig. 4.13C).

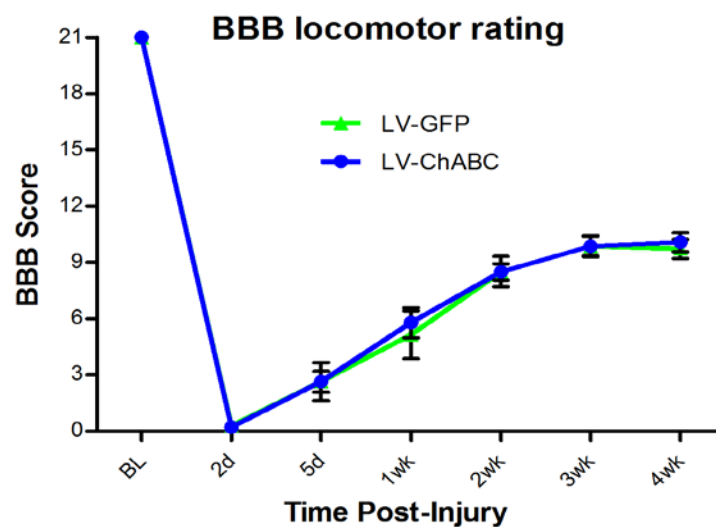


Figure 4.11. Treatment of a severe thoracic contusion injury with LV-ChABC does not lead to improvements in BBB locomotor scores. Assessment of gross locomotor function using the BBB scoring system shows that both LV-ChABC and LV-GFP treated animals follow a similar pattern of impairment and spontaneous recovery following a severe thoracic contusion injury. n=7 per group.

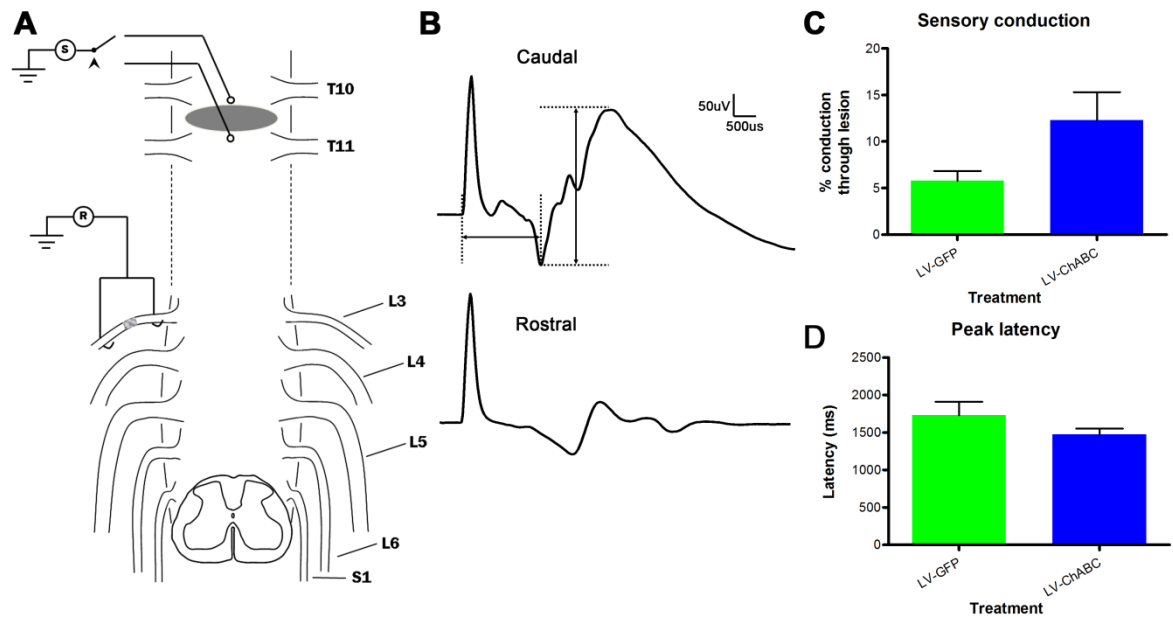


Figure 4.12. Conduction of the ascending sensory pathway through a severe thoracic contusion injury is improved in LV-ChABC treated animals. (A) Schematic representation of the electrophysiological protocol used to assess dorsal column sensory conduction through a severe thoracic contusion injury. At 4 weeks post-injury sensory compound action potentials (SCAPs) were recorded from whole dorsal roots (R) whilst first stimulating (S) caudal to the lesion site and then rostral. The amplitude of the response elicited following stimulation rostral to the lesion was quantified as a percentage of the amplitude of the response elicited following caudal stimulation. The dorsal root was crushed in between the anode and cathode of the recording electrode. (B) Example traces recorded from an L6 dorsal root in an LV-ChABC treated animal following rostral and caudal stimulation at 4 weeks post-injury. A vertical arrow on the “caudal” trace indicates how peak-to-peak amplitude was measured, whilst a horizontal arrow indicates how peak latency was measured. (C) Quantification of SCAP amplitudes shows that the mean amplitude of SCAPs recorded in LV-ChABC treated animals is over double that observed in LV-GFP treated animals ($12.3\% \pm 3$ vs $5.5\% \pm 1.6$; $p=0.07$, unpaired t-test). (D) Quantification of peak latencies shows that LV-ChABC treated animals have a slightly shorter latency than those treated with LV-GFP, suggesting that conduction through the injury site may be slightly quicker in these animals. $n=4$ per group.

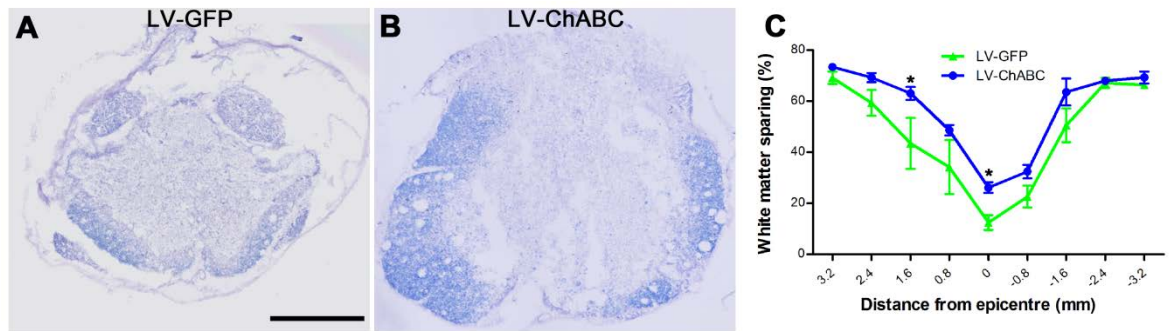


Figure 4.13. LV-ChABC treatment significantly enhances white matter sparing following severe contusion injury. Representative images of injury epicentre from LV-GFP (A) and LV-ChABC (B) treated animals. (C) Quantification of white matter throughout the injured area of the spinal cord reveals a significant sparing of white matter at lesion epicentre, as well as a reduction in rostro-caudal spread of white matter damage, following treatment with LV-ChABC (* $p < 0.05$, two-way RM ANOVA, $n = 4$ per group). Scale bar = 500 μ m.

4.4 Discussion

The results of this chapter demonstrate the efficacy of ChABC gene therapy in promoting functional repair in two clinically relevant models of SCI (moderate cervical contusion and severe thoracic contusion), thereby emphasising its therapeutic potential as a treatment for SCI. Firstly, a range of behavioural techniques were used to carry out a detailed assessment of post-injury functional changes in both male and female rats following a moderate cervical contusion injury and to determine the optimal tests for assessing the efficacy of therapeutic intervention in this model. Using these behavioural tests, combined with electrophysiological assessments, the efficacy of LV-ChABC to promote functional repair in this injury model was then assessed. Behavioural assessments revealed improvements in skilled locomotion and a small increase in forelimb grip-strength following LV-ChABC treatment, whilst electrophysiological assessments demonstrated improved functionality of specific spinal pathways. These changes were associated with improvements in tissue preservation in LV-ChABC treated animals. Finally, LV-ChABC treatment was also shown to lead to improved conduction of dorsal column sensory fibres through a severe thoracic contusion injury and this was associated with enhanced sparing of white matter throughout the contusion injury site. Whilst the findings of this chapter indicate that the beneficial effects of LV-ChABC observed in the previous chapter can be replicated in contusion injury models of varying severity or spinal level, a number of these findings were made in small sample sizes and remain preliminary due to this being an ongoing study. Plans for further experiments will be discussed in section 4.4.5.

4.4.1 Functional changes following a cervical contusion injury

A number of different behavioural tests were utilised in study one of this chapter in order to assess different aspects of spontaneous functional recovery following a cervical contusion, as well as to evaluate which behavioural tests would be most useful for a larger study assessing the effects of LV-ChABC in this injury model. A number of different spinal pathways contribute to the behaviours involved in each of these tasks (Soblosky et al., 2001;Sedy et al., 2008;Sundberg et al., 2010), so using a wide variety of behavioural assessments provides functional data on a larger sample of different pathways. For example, the inclined plane primarily assesses proprioceptive function and balance. Both vestibulospinal and reticulospinal tract function have been shown to be particularly important for this task (Fehlings and Tator, 1995;Deliagina et al., 2006;Sedy et al., 2008), whilst CST function has been shown to have minimal influence (Fehlings and Tator, 1995). The horizontal ladder task, on the other hand, assesses skilled locomotion and requires the integration of sensory and motor function and therefore is affected by the function of the ascending dorsal column sensory pathway, corticospinal, reticulospinal and rubrospinal tracts (Metz et al., 2000b;Metz and Whishaw, 2002;Sedy et al., 2008;Sundberg et al., 2010). Ultimately the most suitable tests were selected based on inter- and intra-animal consistency in results and the extent to which each test highlighted a functional deficit post-injury and portrayed spontaneous recovery, but also ensuring that a wide variety of functions and spinal pathways were still being assessed. The hanging test did not show any particular pattern of post-injury deficit or recovery and therefore was not selected. Additionally, the sticky tape removal task showed high levels of inter-animal variability and therefore, it too, was not selected. In general, the functional data obtained using these behavioural tests closely matched the pattern of post-injury functional changes observed in previous studies

characterising cervical contusion injuries (Pearse et al., 2005;Anderson et al., 2009a;Anderson et al., 2009b;Aguilar and Steward, 2010), highlighting the reproducibility of this injury model.

Digigait analysis provided useful information on a number of gait parameters that were notably affected by cervical injury. Both forelimb and hindlimb parameters were affected by injury, but interestingly forelimb deficits appeared to be more substantial. The fact that the contusion injury level was at C5 means that there is likely to have been a substantial loss of motor neurons important for forelimb motor function. Indeed, previous findings using an injury model of similar severity to this study showed a complete loss of ventral horn neuronal cell bodies at the lesion epicentre and further neuronal losses spreading for ~1mm either side of the epicentre (Pearse et al., 2005). It is therefore not entirely surprising that forelimb function is more affected by this injury than hindlimb function. It is interesting however, that locomotor scoring using the FLS did not indicate any differences in forelimb or hindlimb function between treatment groups, with both recovering to a score of 15 by 4 weeks post-injury. This again highlights the importance of using a variety of functional assessments, as some tests may not be sensitive to particular aspects of impairment or recovery. The slight disparity in severity of impairment between forelimbs and hindlimbs is also likely to have contributed to the observed deficits in gait symmetry. Digigait provided useful quantitative analysis that highlighted additional deficits that could not necessarily be detected during observational behavioural tests. Unfortunately, the inability to coerce many of the male rats into running on the treadmill, particularly post-injury, meant that Digigait analysis could not be used in study two of this chapter (treatment of a cervical contusion).

4.4.2 Electrophysiologically detected changes in spinal pathway functionality following LV-ChABC treatment of a cervical contusion

The finding that treatment with LV-ChABC led to significant improvements in the conduction of long-distance sensory axons through the cervical contusion injury site substantiates similar findings in the preceding chapter. That LV-ChABC treatment leads to improvements in sensory conduction following a contusion injury at either thoracic or cervical level is a particularly promising finding, as efficacy of any potential treatment following injury at different spinal levels significantly enhances its therapeutic potential. The fact that in both instances these improvements were seen in clinically relevant contusion injury models is also important when considering the translatability of LV-ChABC to the clinical situation. As discussed in the previous chapter (section 3.4.2), these improvements are likely due to enhanced axonal sparing and alleviation of CSPG-induced conduction block (Hunanyan et al., 2010;James et al., 2011)

Whilst the finding of improved sensory conduction repeated what had previously been seen following thoracic contusion injury, the electrophysiological assessment of CST function provided novel findings on the effects of ChABC gene therapy. A number of previous studies have shown that ChABC enzyme can lead to regeneration, plasticity and functional improvements in the injured CST (Bradbury et al., 2002;Garcia-Alias et al., 2008;Nakamae et al., 2009;Garcia-Alias et al., 2009b;Wang et al., 2011a;Starkey et al., 2012). Bradbury *et al.* (2002) found that, following a dorsal column crush injury (including the main component of the CST), repeated intrathecal infusions of ChABC resulted in collateral sprouting of CST fibres rostral to the lesion site, a small number of CST fibres regenerating through the lesion site and improved functionality of the CST, as assessed using the same electrophysiological technique used in this chapter. Another study showed that ChABC treatment following discrete unilateral transection of the

CST led to significant sprouting of the intact CST, much of which crossed the midline to the denervated side where it was associated with the presynaptic marker vGlut1 (Starkey et al., 2012). This plasticity was associated with functional recovery in the affected forelimb. Whilst the electrophysiologically assessed improvements in CST functionality in the current study were not significant, the fact that the mean amplitude of the CST response in LV-ChABC treatment animals was double that observed in control groups, along with previous findings on the effects of ChABC on the CST, suggests that LV-ChABC treatment is likely to be having effects on the CST. As seen following anatomical assessment of the lesion, the main component of the CST (located at the base of the dorsal columns) is entirely abolished following cervical contusion (Fig. 4.5+4.9). It therefore seems likely that any improvements in CST function are due to plasticity, particularly in light of previous findings that ChABC can induce plasticity in a number of spinal pathways, including the CST (Barritt et al., 2006;Massey et al., 2006;Cafferty et al., 2008;Starkey et al., 2012). In addition, results from the previous chapter highlight the effects of LV-ChABC on plasticity. The minor components of the CST are located in the dorsolateral and ventral white matter and, therefore, appear to be at least partially spared in this injury model (Fig. 4.5+4.9), suggesting that enhanced CST functionality could result from collateral sprouting of the main component of the CST rostral to the lesion and/or compensatory activity and sprouting of the minor CST components. As in the case of improved sensory conduction, the alleviation of CSPG-mediated conduction block may also play a role in improved CST functionality (Hunanyan et al., 2010;Petrosyan et al., 2013).

4.4.3 Improvements in behavioural function following LV-ChABC treatment of a cervical contusion

Whilst a number of different behavioural tests were used in order to assess the effects of LV-ChABC on functional recovery following a cervical contusion injury, functional improvements were primarily detected using the horizontal ladder task. This suggests that LV-ChABC may affect some pathways differently to others and lead to functional improvements particularly in tasks that rely on the function of pathways it affects the most. The fact that electrophysiological improvements were observed in both the CST and the ascending sensory pathway adds credibility to this hypothesis, as both of these pathways are heavily involved in skilled locomotor function (Metz et al., 2000b; Metz and Whishaw, 2002; Sedy et al., 2008). Furthermore, neither of these pathways is thought to be important to performance on the inclined plane task (Fehlings and Tator, 1995; Deliagina et al., 2006; Sundberg et al., 2010) and LV-ChABC treatment was not associated with any improvements in this task. This suggests that, whilst LV-ChABC treatment led to improvements in CST and sensory pathway function, it may have had little effect on pathways important in inclined plane performance, such as the vestibulospinal and reticulospinal tracts (Fehlings and Tator, 1995; Deliagina et al., 2006). Forelimb grip-strength, as with the horizontal ladder, is thought to partially relate to CST function (Pearse et al., 2005; Anderson et al., 2009b), whilst sensory function can also affect performance in this task, as animals with severe sensory impairments will not grasp the grip-strength bar (Anderson et al., 2009b). Additionally, as this test assesses neuromuscular function, the loss/preservation of motoneurons innervating the forelimb will also contribute to performance in this task. The fact that LV-ChABC treatment led to enhanced tissue sparing suggests that increased survival of these motoneurons may also play a role in improved forelimb grip-strength. Although no

functional improvements were detected using the FLS scoring system, the fact that Digigait analysis highlighted a number of locomotor deficits that could not be detected by this observational locomotor scoring (particularly a disparity in forelimb and hindlimb deficits), suggests that perhaps this test is not particularly sensitive for a number of aspects of locomotor function.

4.4.4 Effects of LV-ChABC in a model of severe contusion

Whilst the effects of LV-ChABC on enhanced sensory conduction and tissue preservation in a severe thoracic contusion injury model were consistent with the previous findings of this thesis, it had been hoped that by increasing the severity of the injury model an effect of LV-ChABC on gross locomotor function would be detected using the BBB scoring system; unfortunately this was not the case. This may be partially attributable to the fact that the severe contusion injury model did not lead to as significant a permanent deficit as had been hoped for. Previous studies showing a treatment leading to improved BBB scores following a contusion injury have normally used any injury severity which results in animals only achieving BBB scores of 4-6 if untreated, whilst treatments have generally resulted in improvements to a score of 9 or 10 (Caggiano et al., 2005; Pedram et al., 2010; Figueroa et al., 2012). In the present study both control and LV-ChABC treated animals recovered to a BBB score of 10, potentially making it more difficult for the BBB scale to detect any improvements (discussed in section 3.4.2). However, the fact that no improvements were detected using this test, or the FLS in the cervical contusion study, does indicate a potential limitation of using LV-ChABC treatment alone. Interestingly the modest effects of LV-ChABC in some outcome measures may also be associated with the lower titer of the batch of LV-ChABC used in this chapter compared with the previous chapter (284

µg/ml compared to 479 µg/ml of P24). Ultimately, ChABC gene therapy may be most effective in combination with an additional therapeutic strategy. The combination of LV-ChABC with rehabilitative techniques seems likely to be a particularly promising combination. Rehabilitative strategies have been shown to promote plasticity and compensatory activity (Girgis et al., 2007; Courtine et al., 2008; Edgerton et al., 2008; Starkey et al., 2011; van den Brand et al., 2012), as well as to guide and refine any functional improvements resulting from an experimental therapeutic, particularly improvements mediated via plasticity, leading to additive or even synergistic effects of combination (Simonen et al., 2003; Garcia-Alias et al., 2009b; Fouad and Tetzlaff, 2012; Garcia-Alias and Fawcett, 2012). As ChABC gene therapy was shown to enhance plasticity in the previous chapter, it seems likely that it may be a good candidate for combination with some form of rehabilitative strategy. Indeed, the combination of ChABC enzyme with behavioural rehabilitation has previously been shown to have additive effects on functional recovery (Garcia-Alias et al., 2009b; Wang et al., 2011a). The widespread and sustained CSPG degradation that is achieved using LV-ChABC is likely to further enhance the beneficial effects that rehabilitation can have in combination with ChABC-mediated CSPG modification.

4.4.5 Future experiments

The work described in this chapter is part of an ongoing study and as such a number of further experiments, designed to further explore a number of the findings as well as enhance their reliability, are yet to be carried out. Initially, a number of further histological assessments will be carried out on the tissue harvested from the animals in each of the three studies described in this chapter. This will include quantification of neuronal survival through the injury site using a marker for neuronal cell bodies

(NeuN), assessment of astrogliosis (GFAP) to determine whether the phenotypic differences in glial scar formation associated with LV-ChABC treatment observed in chapter three are repeated, analysis of the extent of CSPG degradation (C-4-S) and if this is associated with any indication of anatomical plasticity (e.g. enhanced 5-HT immunoreactivity) and further tissue sparing analysis will be carried out in order to increase the sample size of data currently presented. Additionally, tissue biopsies from various forelimb and hindlimb muscles in each animal were harvested and snap frozen prior to transcardial perfusion to allow for assessment of injury and/or treatment induced changes in muscle fibre morphology and composition. This will be particularly interesting to assess whether there are any detectable changes to forelimb muscles, such as a decrease in average fibre diameter or a change in the ratio of type I to type II fibres, and if these are correlated with the observed changes in forelimb grip-strength in particular.

A number of further experiments will also be carried out. Firstly, an additional cohort of animals will be set up to repeat the electrophysiological assessment of CST function carried out in study two. The numbers involved in the initial assessment were very small (n=3 per group) and variability of the recordings was very high, so sample sizes for this particular aspect of the study need to be increased to improve the reliability of the findings. Additionally, following the initial assessment of CST function in a number of these animals, a discrete lesion of the CST will be performed at the level of the brainstem and function re-assessed to ensure that the recordings attributed to the CST are then ablated.. The whole of study three will be repeated using a further increased injury severity over a longer time course in order to potentially reveal a difference in locomotor recovery at the lower end of the BBB scale as well as to further validate the findings presented in this chapter. Thus, whilst a number of the findings in

this chapter are based on preliminary observations, extensive further work is ongoing that will enhance the validity of these findings as well as producing additional data, potentially revealing further effects of LV-ChABC.

Chapter Five

5 Chapter 5:

General Discussion

5.1 Summary of findings

This thesis has used clinically relevant spinal contusion injury models of differing levels and severities in order to assess the therapeutic potential of ChABC gene delivery as a treatment for SCI. A detailed injury characterisation was performed in chapter two, examining the temporal pattern of functional and pathological changes that take place following a thoracic contusion injury of moderate severity in rats. In addition, a novel electrophysiological technique used to examine the conduction properties of single sensory fibres through the contusion injury site was also established in this chapter. Behavioural techniques depicted the time course of changes in both skilled and gross locomotor function, highlighting the spontaneous recovery that occurs over sub-acute time points, but that animals remain substantially impaired in the chronic post-injury stage. The novel electrophysiological technique revealed acute conduction failure in the ascending sensory fibres of the dorsal columns. Conduction was gradually restored in some axons over the sub-acute to chronic post-injury phase, but remained impaired as indicated by reduced conduction velocities. Cooling of the lesion site revealed a population of spared, viable axons unable to conduct under normal physiological conditions which may correspond to chronically demyelinated axons observed using electron microscopy.

Chapter three went on to assess the efficacy of ChABC delivery via a lentiviral vector when used to treat the thoracic contusion injury model characterised in chapter two. Intraspinal injections of LV-ChABC at the injury site resulted in sustained, widespread degradation of CSPGs which was associated with significant improvements in functional and anatomical outcome measures. Histological analyses depicted dramatic improvements in post-injury pathology following ChABC gene therapy, whilst behavioural analyses highlighted significant functional improvements in skilled locomotor function. The novel electrophysiology technique established in chapter two was utilised to reveal enhanced conduction of long distance sensory fibres through the injury site. Long-term and widespread CSPG degradation also resulted in enhanced anatomical and functional plasticity below the level of the injury. Finally, preliminary observations indicated that LV-ChABC treatment was associated with modulation of the post-injury inflammatory response towards a reparatory phenotype.

The therapeutic potential of ChABC gene therapy was further assessed in chapter four using a cervical contusion injury model and a more severe model of thoracic contusion injury. Improvements in sensory fibre conduction through the injury site were detected in both injury models and in both instances this was associated with enhanced tissue preservation. Further electrophysiological analysis in the cervical injury model also demonstrated improved functionality of the CST caudal to the contusion injury. Finally, behavioural assessment of cervically contused animals revealed significant improvements in skilled forelimb and hindlimb locomotor function as well as increased forelimb grip-strength in LV-ChABC treated animals.

Thus, work in this thesis has established a novel electrophysiological technique that can be used to assess the conduction properties of individual sensory fibres in the injured spinal cord, demonstrated efficacy of ChABC gene therapy in promoting

functional and anatomical repair in a variety of clinically relevant contusion injury models, and revealed that these effects are likely to be primarily due to the neuroprotective and plasticity-enhancing effects of ChABC delivered via viral vector.

5.2 Potential mechanisms underlying the effects of ChABC

Extensive research now exists demonstrating the efficacy of ChABC as a potential therapeutic for SCI using a variety of injury models as well as different animal models (Bradbury et al., 2002;Caggiano et al., 2005;Massey et al., 2006;Cafferty et al., 2008;Carter et al., 2008;Tester and Howland, 2008;Filous et al., 2010;Starkey et al., 2012). Whilst the beneficial effects of ChABC were initially attributed to its effects on promoting regeneration (Yick et al., 2000;Bradbury et al., 2002;Yick et al., 2003), it has since additionally been shown to promote neuroplasticity (Barritt et al., 2006;Massey et al., 2006;Galtrey et al., 2007;Cafferty et al., 2008;Tom et al., 2009a;Starkey et al., 2012) and have neuroprotective effects (Carter et al., 2008;Carter et al., 2011). Recently it has also been suggested that ChABC can promote migration, differentiation and process outgrowth of OPCs and adult oligodendrocyte cells, potentially enhancing post-injury remyelination (Siebert and Osterhout, 2011;Siebert et al., 2011;Pendleton et al., 2013). Thus, the beneficial effects of ChABC are multifaceted, combining to make it one of the most promising potential therapeutics currently being developed for the treatment of SCI. However, before any experimental therapeutic can be considered for use in humans, it would be greatly advantageous if there is a reasonable understanding of the underlying mechanisms mediating the beneficial effects of any treatment.

The multifaceted nature of the beneficial effects of ChABC indicates that the mechanisms through which they are mediated are likely to be numerous and complex. Whilst the initial enzymatic cleavage of CS-GAGs from the CSPG core protein by ChABC is a simple and straight-forward mechanism of action, the complex interactions

of intact CSPGs and their CS-GAG side chains with their surrounding environment means that their degradation results in a wide array of knock on effects. Many of these effects are due to the loss of binding sites for a number of factors present on CS-GAGs following their enzymatic removal. The competitive binding of CSPGs to growth permissive substrates, such as laminin and NCAM, is via binding sites situated on CS-GAGs (Zuo et al., 1998a;Zuo et al., 1998b;Sasaki et al., 2001). Therefore, the removal of CS-GAGs frees up these binding sites, allowing the binding of axons to growth permissive substrates. This binding is mediated via membrane bound integrins, which serve as transmembrane receptors for molecules in the ECM, transducing extrinsic cues and leading to modulation of axon growth (Hynes, 2002;Myers et al., 2011;Eva et al., 2012;Sharma et al., 2012). Binding of integrins to growth permissive ECM substrates, such as laminin, results in positive regulation of growth and allows axonal growth through the ECM (Myers et al., 2011). However, binding to CSPGs inactivates integrin receptors, preventing such interactions. Conversely, integrin overexpression or activation has been shown to promote axon growth over inhibitory CSPGs (Condic, 2001;Tan et al., 2011). Whilst the effect of ChABC mediated CSPG degradation on integrin activation is yet to be specifically investigated, it seems likely that prevention of CSPG interactions with growth permissive substrates and integrin receptors is one mechanism through which ChABC exerts its positive effects on axonal sprouting and regeneration.

In addition to preventing indirect inhibitory actions of CSPGs, ChABC prevents the binding of CSPGs to its specific receptors; LAR and PTP σ (Sharma et al., 2012). The binding sites for these receptors are also located on the CS-GAG side chains (Shen et al., 2009;Fry et al., 2010;Fisher et al., 2011;Sharma et al., 2012). Enzymatic cleavage of CS-GAGs therefore prevents CSPG-induced axonal repulsion and growth cone

collapse mediated via these receptors. Whilst these actions of ChABC alleviate much of the inhibition exerted by CSPGs, the removal of CS-GAGs can also potentially lead to an increased regenerative response in CNS neurons. The GAGs of both CSPGs and HSPGs (both of which are enzymatically cleaved by ChABC) are known to bind growth factors (Deepa et al., 2002;Nandini et al., 2004;Nandini and Sugahara, 2006). Hydrolysis of CSPGs and HSPGs by ChABC will likely render any previously bound growth factors accessible to CNS axons, whether injured or not, promoting axonal outgrowth and neuronal survival. Release of previously GAG-bound growth factors could, therefore, contribute to the processes of regeneration, plasticity and neuroprotection. The functional effects of regeneration and plasticity would be limited however, if it were not for the degradation of PNNs around neuronal cell bodies. Disruption of PNNs by ChABC means that sprouting or regenerating axons are able to form functional synapses on target cells previously surrounded by inhibitory PNNs (Pizzorusso et al., 2002;Massey et al., 2006;Pizzorusso et al., 2006). In addition to disinhibition of synaptogenesis, the degradation of PNNs by ChABC has been shown to result in short-term synaptic plasticity, due to the increased mobility and exchange of AMPA receptors (Frischknecht et al., 2009), and can also result in long-term synaptic plasticity due to enhanced structural and functional plasticity of dendritic spines (de Vivo et al., 2013). This indicates that there are numerous mechanisms through which ChABC can enhance levels of CNS plasticity. The enhanced formation of functional connections by injured axons due to PNN degradation could also lead to neuroprotective effects, reversing any atrophy initially caused by axotomy and the associated lack of trophic support as well as potentially preventing the gradual apoptosis of axotomised neurons (Barron et al., 1988;Giehl and Tetzlaff, 1996;Bradbury et al., 1998;Barron, 2004;Wannier et al., 2005). Another mechanism potentially contributing to the

neuroprotective effects of ChABC is the attenuation of macrophage-induced axonal die-back following CSPG degradation, as shown *in vitro* (Busch et al., 2009). This attenuation of axonal die-back may well be due to the change in macrophage phenotype following ChABC treatment observed in chapter three. Particularly as modulation of the immune response in this way has previously been shown to enhance CNS repair and limit inflammatory-mediated damage (Kigerl et al., 2009;Shechter et al., 2009;Donnelly et al., 2011;Shechter et al., 2013). Therefore, if further experiments can substantiate and reveal a mechanism for the observed association between ChABC-mediated CSPG degradation and immunomodulation, this could be a novel mechanism underlying the beneficial effects of ChABC. Thus, there are numerous and complex mechanisms underlying the effects of ChABC on regeneration, plasticity and neuroprotection already proposed or known and it is likely that there are many more yet to be discovered.

In addition to its effects on regeneration, plasticity and neuroprotection, ChABC can also promote functional improvements through alleviation of CSPG-induced conduction block. The CSPG NG2 has been shown to induce conduction block in spinal axons following its exogenous application and displays a post-injury pattern of accumulation at the nodes of Ranvier (Hunanyan et al., 2010). ChABC treatment was shown to restore conduction in surviving axons passing through areas of high CSPG levels (Hunanyan et al., 2010) and an NG2 antibody was shown to restore conduction in many spared axons following a hemisection injury (Petrosyan et al., 2013). If a significant number of axons are affected by CSPG-induced conduction block following injury then alleviation of this may well lead to significant functional improvements; indeed, it is entirely feasible that this mechanism contributed towards many of the electrophysiological changes observed throughout this thesis. One final mechanism which may contribute to the beneficial effects of ChABC is the recently explored effect

of CSPG degradation on oligodendrocyte lineage cells. CSPGs have been shown to be inhibitory to OPC migration and process outgrowth (Siebert and Osterhout, 2011; Siebert et al., 2011) and these effects have been shown to be mediated via the presence of PTP σ on oligodendrocyte lineage cells. ChABC has been shown to prevent these inhibitory effects and reverse CSPG-mediated inhibition of myelination *in vitro* (Pendleton et al., 2013). Therefore, it is possible that ChABC treatment improves oligodendrocyte-mediated remyelination post-injury and that the enhanced migration of OPCs to the lesion site provides additional trophic support for injured and demyelinated axons. It is clear that the actions of ChABC are associated with a wide variety of effects in the injured CNS, many of which have been proposed as potential mechanisms contributing to the enhanced spinal repair associated with ChABC treatment. However, further research is required in order to gain a better understanding of each of these proposed mechanisms and to determine the importance of each in terms of its contribution to functional repair. Further understanding will also aid in the development of more specifically targeted therapies. For instance, the identification of the CS-E sulphation motif as a key site for many of inhibitory interactions of CSPGs has led to the development of a synthetic anti-body against this motif which has shown promising effects on regeneration *in vivo* (Gama et al., 2006; Rogers et al., 2011; Brown et al., 2012).

5.3 Issues associated with clinical translation

5.3.1 Translatability of ChABC gene therapy

Although ChABC represents a promising experimental therapeutic there are a number of issues associated with this treatment which would need to be addressed before any

assessment could be carried out in human SCI patients. The delivery of ChABC via gene therapy has significantly improved upon previously used delivery methods in that long-term and widespread CSPG degradation can be achieved following a single administration. Despite the significant advantage this offers, the use of a viral vector as a delivery vehicle is associated with some risks and these would need to be minimised in order to further enhance the clinical translatability of ChABC. Firstly, the LV used throughout this thesis is capable of integrating into the host genome; this carries a potential risk of insertional mutagenesis which could even lead to oncogene activation, although the chances of this are extremely small (Baum and Fehse, 2003; Baum et al., 2003) and no adverse effects were observed in any treated rats even when ChABC was expressed for over a year. The recent development of integration-deficient lentiviral vectors which display efficiency equal to that of integrating vectors (Yanez-Munoz et al., 2006; Peluffo et al., 2013) means that this risk could be overcome by using an integration-deficient LV to deliver the mammalian compatible, engineered ChABC gene. Additionally, although the long-term expression of ChABC following LV-ChABC treatment led to beneficial effects on spinal conduction and plasticity, to be considered for human use such long-term expression would likely need to be controllable in order to minimise safety concerns. This could potentially be achieved with the incorporation of an inducible promoter into the viral vector containing the ChABC gene. The most effective way this has been achieved to date has involved the utilisation of elements of the tetracycline-resistance operon of *E.coli*, such that a tetracycline (TC) responsive element is fused to an activating domain (TC-controlled transactivator) which controls the activity of the promoter controlling transgene expression (Gossen and Bujard, 1992; Gossen et al., 1995; Regulier et al., 2002; Vigna et al., 2002; Blesch et al., 2005). Using this system transgene expression can be regulated

by the administration of the antibiotic tetracycline or its derivatives (e.g. doxycycline). Importantly this family of antibiotics can cross the blood-brain barrier and bind with high affinity to the TC-responsive component incorporated into the LV, so low-dose, oral administration of the antibiotic is sufficient to regulate transgene expression (Gossen et al., 1995;Regulier et al., 2002). Furthermore, this system can be modified so that TC administration will either induce (“tet-on”) (Gossen and Bujard, 1992) or prevent (“tet-off”) (Gossen et al., 1995;Vigna et al., 2002) transgene expression. This is achieved through modification of the TC-responsive transactivator, such that binding of TC can either induce or abolish binding of the TC-responsive transactivator to its specific DNA target sequence which controls promoter activity. In terms of clinical applications, the TC-induced transgene expression system (“tet-on”) is the most viable as this would require administration of antibiotics only during the desired period of treatment (e.g. only 10 weeks of antibiotic administration immediately following LV injection to allow ChABC expression for that period only). Thus, the use of an integration-deficient, TC-inducible lentiviral vector to deliver the engineered ChABC gene could potentially minimise the key issues associated with LV gene therapy. It is also particularly promising that LVs have already been used, or are currently in use, in gene therapy clinical trials (Manilla et al., 2005;Bank et al., 2005;Levine et al., 2006;D'Costa et al., 2009;Cavazzana-Calvo et al., 2010). AAVs are often viewed as a potentially safer option for clinical gene therapy (see section 3.1.2) and therefore, in planned future studies the delivery of the ChABC gene via an AAV will also be assessed in a spinal contusion injury model. It is possible that the slow onset of expression using AAVs may limit the efficacy of this treatment however.

In addition to the potential issues of LV use in patients, the fact that ChABC is a bacterial enzyme suggests it could possibly elicit a negative immune response resulting

in infection and having detrimental effects on recovery. Results in chapter three indicating that LV-ChABC modulates the immune response in a positive way suggest the opposite is true however, at least in rodents. Indeed, disaccharides cleaved from CSPGs have been shown to result in activation of a reparatory phenotype in CNS immune cells *in vitro* (Ebert et al., 2008). Additionally, the ECM degrading enzyme hyaluronidase has been approved for use in humans having shown no adverse effects in clinical testing (Kuppermann et al., 2005). Whilst the clinical version of hyaluronidase (Vitrase) is not of bacterial origin, it does come from a foreign source (ovine) and is not associated with an enhanced rate of infection. It therefore seems that, although there are potential issues with the clinical translatability of ChABC gene therapy, it may be possible to minimise these issues in such a way that they will not present an insurmountable barrier to the clinical assessment of this treatment should it prove efficacious in larger preclinical models of SCI (e.g. non-human primate or porcine).

5.3.2 Past and present spinal cord injury clinical trials

Whilst there are numerous hurdles to overcome before any experimental therapeutic can make the leap forward from an experimental treatment into being assessed in the clinical setting, a number of treatments which have displayed promising results in the pre-clinical setting have undergone efficacy testing in human clinical trials. The majority of treatments that have reached the stage of clinical trials fall under three broad categories; neuroprotective therapeutics, regeneration-promoting therapeutics, and cell-based strategies (Gensel et al., 2011;Varma et al., 2013). One neuroprotective therapeutic, methylprednisolone, is the only treatment to have gone through extensive clinical testing (Bracken et al., 1984;Bracken et al., 1990;Bracken et al., 1997) and to have subsequently been recommended for clinical use in a number of countries. However, as previously discussed (section 1.1), the beneficial effects of

methylprednisolone are highly controversial and the high doses required have been linked to increased incidence of infection and respiratory complications (Hurlbert, 2000;Short et al., 2000;Hurlbert, 2001;Matsumoto et al., 2001). Accordingly it is now no longer a “standard of care” for treatment of acute SCI in the majority of the countries in which this was initially the case (Felleiter et al., 2012;Breslin and Agrawal, 2012;Druschel et al., 2013) (<http://www.surgicalcriticalcare.net/Guidelines/methylprednisolone.pdf>). A number of other additional neuroprotective therapeutics have been assessed or are currently being assessed in clinical trials. Of the neuroprotective treatments to complete clinical trials, none have been found to lead to significant clinical benefits as of yet (Gensel et al., 2011;Varma et al., 2013). For example: Nimidopine (a calcium channel blocker shown to improve blood flow to the cord and significantly reduce apoptosis in preclinical models) failed to show any benefits in a randomised controlled trial and was associated with increased risk of infection (Pointillart et al., 2000); acute administration of Gacyclidine (an NMDA receptor antagonist shown to reduce glutamate excitotoxicity) failed to show any improvements in ASIA motor and sensory scores or in the functional independence measure at a one year follow up (Fehlings and Baptiste, 2005;Tator, 2006); a small randomised trial was also carried out to assess thyrotropin releasing hormone (Pitts et al., 1995), which has been shown to have neuroprotective effects through its antioxidant and membrane stabilising properties (Faden et al., 1984), and despite patients with incomplete SCI displaying detectable improvements in neurological recovery, a larger follow up trial was never conducted. Whilst none of these previous trials have led to the successful translation of a therapeutic, there are a number of ongoing clinical trials assessing various neuroprotective treatments. Minocycline and erythropoietin both exert neuroprotective effects through their anti-inflammatory actions, whilst Riluzole and

HP184 exert their neuroprotective effects through selective blockade of sodium channels; each of these treatments is either currently undergoing clinical trial or has completed its clinical trial but official findings are yet to be published (clinical trial IDs: NCT00559494 – Minocycline, NCT00561067 – Erythropoietin, NCT00093275 – HP184, NCT00876889 – Riluzole) (Gensel et al., 2011).

In terms of regeneration-promoting therapeutics, only a small number of experimentally developed treatments have thus far reached the stage of human testing. The humanised anti-NogoA antibody (ATI355) has completed a phase I safety trial (NCT00406016). No adverse effects were reported following intrathecal administration in a trial involving 52 patients with spinal injuries of varying levels and severities; unfortunately no findings were reported in regard to observations on neurological changes (Zorner and Schwab, 2010). A phase II clinical trial was scheduled to begin in 2010, this is currently ongoing and as of yet no update on the progress of the trial has been published. One of the largest SCI clinical trials to date, in terms of number of patients recruited, was used to assess the efficacy of the ganglioside GM-1. Gangliosides are complex glycolipids found in the cell membrane at particularly high concentrations in the CNS, and the ganglioside GM-1 was shown to lead to enhanced axonal sprouting *in vitro* (Ferrari et al., 1983) as well regeneration in a number of CNS injury models *in vivo* (Sabel et al., 1984; Cuello et al., 1986; Bose et al., 1986; Sabel et al., 1987). Following safety trials in humans, this led to a large phase II clinical trial involving ~800 patients with SCI. Unfortunately, intravenous dosing of GM-1 led to no significant improvements in any of the primary outcome measures of the trial, although a trend towards improved sensory and bladder/bowel function was reported (Geisler et al., 2001a; Geisler et al., 2001b). Cethrin is another therapeutic targeting regeneration which has undergone clinical trial (NCT00500812 – a phase I/IIa trial to

evaluate safety, tolerability and pharmacokinetics). Cethrin is derived from C3 transferase, a bacterial enzyme which disrupts the Rho signalling pathway by blocking RhoA (Lehmann et al., 1999;Dergham et al., 2002). The Rho pathway has been shown to be involved in both CSPG and myelin induced inhibition (Niederost et al., 2002;Monnier et al., 2003). Upon completion of the phase I/IIa trial, findings reported no serious adverse effects of extradural administration of Cethrin and indicated that treatment led to improved motor recovery (Fehlings et al., 2011). However, the larger phase II trial to assess efficacy which followed has since been terminated (NCT00610337). Finally, a clinical trial was undertaken in order to assess the efficacy of orally administered lithium as a potential treatment for SCI (NCT00750061). Lithium blocks the actions of glycogen synthase kinase 3 β (GSK-3 β), a kinase which has inhibitory effects on axon growth, and has been shown to promote regeneration in experimental SCI (Dill et al., 2008). Whilst no serious adverse effects were reported in an initial phase I trial (NCT00431171), the subsequent phase II trial to assess efficacy reported no improvements in neurological outcomes (Yang et al., 2012).

As has been the case with clinical trials assessing various neuroprotective or regeneration-promoting therapeutics, clinical trials involving cell-based strategies for treatment of SCI have so far met with limited success. Whilst a number of different cell types have been transplanted into SCI patients in trials of varying sizes, stem cells have been the most commonly used cell type in clinical studies with at least five separate human trials taken place involving the transplantation of MSCs (NCT00816803, NCT00695149, NCT01162915, NCT01186679, NCT01274975) (Gensel et al., 2011;Tohda and Kuboyama, 2011;Varma et al., 2013), whilst many more are currently recruiting patients (www.clinicaltrials.gov). The results of these trials have been varied and need to be interpreted with caution as most trials have been small and have been

carried out open-label. In general, MSCs have been administered intrathecally and each study has reported no significant adverse effects, although in one trial spasticity and neuropathic pain were observed in some patients (Kishk et al., 2010). Many of these trials have reported modest sensory and/or motor improvements associated with intrathecal MSC infusion (Sykova et al., 2006;Deda et al., 2008;Kumar et al., 2009;Kishk et al., 2010), although as of yet no larger, randomised and controlled clinical trials have been carried out. Another cell type that has been used in clinical SCI studies is the OEC. Having seen promising results following OEC transplantation in a number of experimental SCI models (Li et al., 1997;Ramon-Cueto et al., 2000;Li et al., 2003), a number of relatively small studies involving the transplantation of OECs into human patients have been carried out (Feron et al., 2005;Lima et al., 2006;Lima et al., 2010b)(NCT01231893) and research has been carried out in order to optimise OEC harvesting protocols from human patients (Choi and Gladwin, 2013;Kachramanoglou et al., 2013). Importantly, no serious adverse effects have been reported in any of these studies, although, whilst one group has reported seeing improved motor and sensory function in association with OEC treatment (Lima et al., 2006;Lima et al., 2010a) the other group saw no such improvements (Feron et al., 2005).

Although there is yet to be a major success in the development of SCI therapeutics from pre-clinical research to significant efficacy in human patients, the number of experimental treatments now reaching the stage of clinical trials is a promising sign for the future of SCI research and provides genuine hope that we are getting closer to being able to significantly improve the prognosis following SCI; a condition once thought untreatable and entirely irreversible. While many trials are ongoing, the failure of so many others suggests that perhaps more caution should sometimes be applied before carrying a treatment forward to the stage of clinical testing.

In some instances there has been minimal pre-clinical data showing efficacy of a therapeutic before it has then been assessed in human patients. For example, there is minimal pre-clinical data on the effects of lithium post-SCI and this treatment was carried forward to human SCI trials despite there only being one *in vivo* study assessing the effects of lithium alone as a treatment for SCI, and this study did not even assess functional recovery (Dill et al., 2008). Prior to any clinical assessment of a therapeutic, the potential treatment should have been robustly assessed in the pre-clinical setting for efficacy. In order for this to be achieved and to maximise the chances of a treatments success in clinical testing, efficacy should be assessed in the most-clinically relevant injury models available (e.g. contusion or compression injury models) and should progress from small, common animal models (e.g. rodents) to larger, more clinically relevant animal models (e.g. non-human primate or porcine), before progressing further to assessment in human patients. If a therapeutic has not displayed robust efficacy in pre-clinical assessment, it is unlikely that it will ever show efficacy in the clinical setting. Additionally, if a potential therapeutic is rushed through to clinical assessment without having first been fully optimised through pre-clinical testing, not only are its chances of success diminished, but its failure will significantly harm the chances of any related therapeutic being taken to clinical trial in the future, even if significant improvements have been made to the therapeutic. Clinical trials are of vital importance to any biomedical research and the growing number of SCI clinical trials is testament to the progress that has been made in this field over a few short decades. However, caution and thorough assessment must also be exercised prior to clinical assessment in order to prevent detrimental effects on future SCI research.

5.4 General conclusions

In conclusion, this thesis aims to emphasise the importance of using clinically relevant injury models in order to improve the translatability of experimental therapeutics for the treatment of SCI. This is not to detract from the importance of other injury models; each injury model can play a vital role in furthering our understanding of SCI, assessing efficacy of experimental therapeutics and/or elucidating the mechanisms underlying any observed efficacy. For example unilateral pyramidotomy is a particularly useful model for assessing either spontaneous or treatment-induced plasticity, complete transection can be used in order to assess the presence of true regeneration and discrete lesions of specific spinal tracts reveals important information about how each individual tract responds to injury as well as how it contributes to function. Since a key aim of this thesis was to optimise and improve the translatability of chondroitinase ABC treatment, a contusion injury model in rats was the clinically relevant injury model used throughout, with spinal contusion injuries representing the most common form of SCI in humans. ChABC therapy was improved in terms of efficacy and translatability via the use of a gene delivery system which allows sustained, widespread delivery of ChABC following a single administration, thereby minimising the invasiveness and risk of infection associated with repeated delivery paradigms. ChABC delivered via a lentiviral vector proved efficacious in a number of different contusion injury models, suggesting that the effects of this treatment are robust and further enhancing its potential translatability. Through the work described in this thesis ChABC gene therapy emerges as a promising therapeutic with the potential for clinical translation. However, further work remains necessary in order to minimise safety issues associated with lentiviral vector-mediated gene therapy as well as the further assessment of LV-ChABC efficacy in larger animal models before clinical translation could be considered. Additionally,

the efficacy of ChABC gene therapy is likely to be significantly enhanced if it can be successfully combined with a complimentary intervention. Therefore, further work should be carried out to assess the therapeutic potential of LV-ChABC in combination with other promising interventions for the treatment of SCI.

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